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(54) METHOD FOR CONTROLLING CLEAVAGE BY OmpT PROTEASE

(57) By clarifying the properties of OmpT protease, a novel use thereof as a protease and a novel method of producing a target polypeptide are provided. More particularly, the present invention relates to a method of controlling cleavage of a polypeptide by OmpT protease which comprises converting a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of said site in said polypeptide into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position concerning said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that desired parts of said polypeptide are cleaved by OmpT protease and/or undesired parts of said polypeptide are not cleaved by ompT protease. For example, the present invention provides a method of treating by OmpT protease a fusion protein having an amino acid sequence wherein the amino acid at the +1-position is X (wherein X is an amino acid other than glutamic acid, aspartic acid or proline); and a method of controlling cleavage by converting the amino acids at the -6-position and the -4-position concerning the cleavage site into amino acids other than acidic amino acids.

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Description

FIELD OF THE INVENTION

[0001] This invention relates to a method of controlling cleavage of a polypeptide by OmpT protease by applying findings on novel cleavage and recognition sites which have been found by examining the substrate specificity of Escherichia coli OmpT protease.

[0002] In one aspect, the present invention relates to a method of cleaving polypeptides by using OmpT protease. More particularly, it relates to a method of cleaving polypeptides by utilizing novel cleavage and recognition sites of OmpT protease.

[0003] In another aspect, the present invention relates to a method of excising physiologically active peptides, proteins and derivatives thereof from fusion proteins with the use of OmpT protease. More particularly, it relates to a method of efficiently producing physiologically active peptides, a protein and derivatives thereof from fusion proteins by examining the substrate specificity of OmpT protease, thus finding a novel cleavage method and cleavage and recognition sites and then utilizing the properties.

[0004] The present invention further relates to a method of avoiding cleavage of polypeptides by OmpT protease at undesired sites. In particular, it relates to a method of avoiding cleavage of physiologically active peptides, proteins or derivatives thereof by OmpT protease in the case of being produced by host cells. That is to say, the present invention provides a method of making the physiologically active peptides, proteins or derivatives thereof not (or hardly) cleavable by OmpT protease by converting the amino acid sequences at the OmpT protease cleavage sites or in the vicinity thereof.

PRIOR ART

[0005] *E. coli* OmpT protease is a protease which exists in *E. coli* outer membrane fraction and selectively cleaves mainly bonds between basic amino acid pairs (Sugimura, K. and Nishihara, T. J. Bacteriol. 170: 5625-5632, 1988). This enzyme has a molecular weight of 36,000 and seemingly falls within the category of serine proteases. Sugimura et al. examined the substrate specificity of this OmpT protease and reported that the enzyme specifically cleaves the peptide bonds at the center of basic amino acid pairs of arginine-arginine, lysine-lysine, arginine-lysine and lysine-arginine. In addition, cleavage sites in amino acid sequences other than the above pairs has been found. Namely, it has been reported that cleavage by OmpT protease arises at arginine-methionine (Zhao, G-P. and Somerville. R. L. J. Biol. Chem. 268, 14912-14920, 1993), arginine-alanine (Lassen, S. F. et al. Biochem. Int. 27: 601-611, 1992) and arginine-valine (Maurer, J. J. Bacteriol. 179: 794-804, 1997). This protease is characterized in that it cleaves not all of proteins and peptides including these sequences but exclusively specific proteins and peptides at specific sites. For example, γ-interferon contains 10 sequences as described above but 2 sequences among them are exclusively cleavable by OmpT protease (Sugimura, K. and Higashi, N. J. Bacteriol.170: 3650-3654, 1988). T7 RNA polymerase contains 17 sequences as described above but 2 sequences among them are exclusively cleavable therewith (Grodberg, J. and Dunn, J. J. J. Bacteriol.170: 1245-1253, 1988). These facts indicate that the above-described data on the cleavage sites of OmpT protease are not applicable to the estimation of its cleavage sites, different from AP-1 and trypsin enzyme which are commonly employed in peptide mapping of proteins and the cleavage sites of which can be estimated on the basis of known data. Since the cleavage by OmpT protease arises at specific sites of proteins or peptides, it is anticipated that amino acid sequences other than the amino acid sequences as described above (namely, the N-terminal and C-terminal amino acid sequences of the cleavage site) may participate in the cleavage. However, it still remains unknown so far what amino acid sequence allows (or does not allow) the cleavage.

[0006] However, OmpT protease has found use in excising target polypeptides from fusion proteins constructed by gene recombination techniques, since it has high specificity to cleavage sites and is one of endogenous proteases of *E. coli*. To release and produce cholesterol esterase by using *E. coli*, Hanke et al. succeeded that the esterase was fused with *E. coli* hemolysin A protein and the fusion protein was released out of cells, and then the protein was treated by OmpT protease on the outer membrane to thereby successfully obtain active cholesterol esterase from the fusion protein. Hanke et al. employed a linker having an arginine-lysine sequence and cleaved this sequence by OmpT protease (Hanke, C et. al. Mol. Gen Genet.233: 42-48, 1992).

[0007] The present inventors found that OmpT protease is resistant to denaturing agents and they clarified that fusion proteins expressed as inclusion body can be cleaved in the presence of a denaturing agent by taking advantage of the above property. Namely, the present inventors successfully produced V8 protease derivative having the enzymatic activity by expressing S. aureus V8 protease derivative fusion protein as inclusion body in an *E. coli* expression system, solubilizing the same by urea, then releasing the V8 protease derivative moiety from the fusion protein by using OmpT protease in the presence of urea and finally refolding (Yabuta, M., Ochi, N. and Ohsuye, K. Appl. Microbiol. Biotechnol.44:118-125, 1995).

[0008] To release target peptides or target proteins from fusion proteins, it has been a practice to employ enzymes having high specificity to amino acid sequences. Known examples of proteases employed for this purpose include Xa factor, thrombin, enterokinase and the like which are enzymes originating in mammals and supplied only in a small amount at a high cost. Therefore, these enzymes are unsuitable for the industrial treatment of peptides and proteins by the fusion protein method on a mass scale. When the target peptide or protein is to be used as a medicine, it is also required to take into consideration viral contamination originating in the enzymes. In contrast thereto, OmpT protease is clearly superior to these enzymes in supply, cost and safety because of originating in *E. coli*.

[0009] However, the substrate specificity of this protease has not been sufficiently studied yet and, therefore, it is difficult at the present stage to arbitrarily design the cleavage site at the desired part to be excised. Moreover, OmpT protease cleaves not all of the sequences reported above (i.e., arginine-arginine, lysine-lysine, arginine-lysine, lysine-arginine, arginine-methionine, arginine-alanine and arginine-valine) but exclusively specific sites in proteins. When one of sequences consisting of these two amino acids is merely located in a linker site of fusion proteins, therefore, this site cannot always be cleaved by OmpT protease. Even though it can be cleaved, OmpT protease cleaves the peptide bond at the center of the cleavage site consisting of two amino acids. Therefore, the amino acid located at the +1-position of the cleavage site will be added to the N-terminus of the target polypeptide when this enzyme cleaves a fusion protein comprising a protective peptide, the amino acid sequence of the cleavage site of OmpT protease and the target polypeptide in this order. Moreover, this added amino acid cannot be arbitrarily selected but restricted to arginine, lysine, valine, alanine or methionine on the basis of the recognition sequences of OmpT protease reported hitherto. These properties of OmpT protease are unfavorable as a protease to be used in cleaving fusion proteins.

[0010] On the other hand, it is known that the cleavage efficiency of papain, which is a protease, is affected not only by the sequence of the cleavage site in the substrate but also by the amino acid sequences in the vicinity thereof (Schechter. I. and Berger, A. Biochem. Biophys. Res. Commun. 27: 157-162 1967). Recently, detailed studies are also made on Kex2 (Rockwell, N. C., Wang, G. T., Krafft, G. A. and Fuller, R. S. Biochemistry 36, 1912-1917 1997) and furin (Krysan, D. J., Rockwell, N. C. and Fuller, R. S. J. Biol. Chem.274, 23229-23234 1999) which are proteases cleaving the C-terminal side of basic amino acid pairs. In Kex2 and furin, the consensus sequences at the cleavage sites and amino acid sequences in the vicinity thereof have been clarified by comparing the amino acid sequences of the substrates. In the case of OmpT protease, it is considered, on the basis of the comparison of the substrates known hitherto, that arginine or lysine is essentially required as the amino acid at the 1-position in the N-terminal side of the cleavage site but no other clear consensus sequence has been found out so far. Although it is presumed that the recognition of the cleavage site and the cleavage efficiency of OmpT protease might be also affected not only by the cleavage site in the substrate but also by the amino acid sequences in the vicinity thereof, it is impossible at the present stage to control the cleavage by OmpT protease by using these properties.

[0011] In the present invention, the location of each amino acid in polypeptides is represented as follows. A sequence site consisting of two arbitrary consecutive amino acids in the polypeptide is referred to as the cleavage site or the site to be cleaved by OmpT protease. Between the amino acids concerning this site, the amino acid in the N-terminal side is referred to as the -1-position while the amino acid in the C-terminal side is referred to as the +1-position. Then the amino acids at the 1st, 2nd, 3rd, and so on in the N-terminal side of this site are referred to respectively as the amino acids at the -1-, -2-, -3-positions and so on, while the amino acids at the 1st, 2nd, 3rd, and so on in the C-terminal side of this site are referred to respectively as the amino acids at the +1-, +2-, +3-positions and so on. When amino acid substitution is introduced into this site or in the vicinity thereof so that the site becomes not cleavable or cleavable, the corresponding amino acids in the sequence are represented by the above-described numbering.

[0012] When an amino acid sequence leucine-tyrosins-lysine-arginine-histidine is to be cleaved at the bond between lysine and arginine (i.e., the two arbitrary consecutive amino acids), for example, leucine, tyrosine, lysine, arginine and histidine serve respectively as the amino acids at the -3-, -2-, -1-, +1- and +2-positions.

SUMMARY OF THE INVENTION

[0013] As described above, OmpT protease is highly useful. When OmpT protease is used as an enzyme cleaving fusion proteins, however, there arise some problems at the present stage such that it is unknown how to design the amino acid sequence at the cleavage site to enable specific cleavage, that the target peptides obtained by the cleavage are restricted due to the restriction on the N-terminal amino acids of the target peptides, and that cleavage cannot be efficiently performed. However it is expected that these problems can be solved by further studying the amino acid sequences at the cleavage site and in the vicinity thereof and establishing a novel cleavage method or novel recognition/cleavage sequences, thereby making OmpT protease further useful in cleaving fusion proteins.

[0014] On the contrary, there sometimes arises a problem of the cleavage by OmpT protease in the production of peptides or proteins by using *E. coli*. The cleavage by OmpT protease may be avoided by, for example, using an OmpT protease-deficient *E. coli* strain as a host or by adding an OmpT protease inhibitor in the steps of the incubation and purification. However, these methods have not been generally employed because such a mutant strain as employed in

the former method is inadequate as a host in some cases, and the addition of enzyme inhibitors in the latter method causes an increase in the production cost or it is feared that the inhibitors might remain in the product. In these cases, moreover, it is impossible to use OmpT protease as an enzyme for cleaving fusion proteins.

[0015] If the cleavage could be avoided by converting amino acid sequence at the undesired cleavage site by OmpT protease or in the vicinity thereof, OmpT protease might be usable as a cleavage enzyme. Therefore it is expected that cleavage can be efficiently avoided while minimizing the conversion of the amino acid sequence by, if possible, clarifying the characteristics of the recognition/cleavage sequences of OmpT protease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

Fig. 1 is a diagrammatic illustration of the construction of pG117S4HR6GLP-1, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; PSRHKR represents a region encoding an amino acid sequence PSRHKR (SEQ ID NO:60); GLP-1[G] represents a region encoding human glucagon-like peptide-1; R6 represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHRWGRSGS (SEQ ID NO:61); Tc^r represents a tetracycline-resistance gene; and lac PO represents *E. coli* lactose promoter operator gene. With respect to pG117S4HGP, see Japanese Laid-Open Patent Publication No. 9-296000 and EP 794255.

Fig. 2 is a diagrammatic illustration of the construction of pG117S4HompRHKR, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; PSRHKR represents a region encoding an amino acid sequence PSRHKR; GLP-1[G] represents a region encoding human glucagon-like peptide-1; R6 represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHRWGRSGS; Tc^r represents a tetracycline-resistance gene; L1 represents an amino acid sequence QMHGYDAELRLYRRHHGSGS (SEQ ID NO:62); and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 3 is a diagrammatic illustration of the construction of pG117S4HompRHPR, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; PSRHKR represents a region encoding an amino acid sequence PSRHKR; GLP-1[G] represents a region encoding human glucagon-like peptide-1; R6 represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHRWGRSGS; Tc^r represents a tetracycline-resistance gene; L1 represents an amino acid sequence QMHGYDAELRLYRRHHGSGS; PSRHPR represents a region encoding an amino acid sequence PSRHPR(SEQ ID NO:63); Linker peptide represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHGSGSPSRHPR (SEQ ID NO:64) wherein L1 is a synthetic DNA ligated to PSRHPR; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 4 shows the whole amino acid sequence of a fusion protein PR encoded by pG117S4HompRHPR, wherein the underlined part corresponds to the amino acid sequence of human glucagon-like peptide-1 (GLP-1[G]); the double-underlined part corresponds to arginine having been converted into another amino acid; and the arrow shows the cleavage site by OmpT protease. The numerical symbols show the amino acid numbers counting from the N-terminus. The protective protein (β-gal117S4H) derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase comprises the amino acid sequence from methionine at the 1-position to arginine at the 127-position. The linker peptide comprises the amino acid sequence from glutamine at the 128-position to arginine at the 153-position. Pre-GLP-1[G] comprises an amino acid sequence from arginine at the +1-position (concerning the cleavage site by OmpT protease) to glycine at the 184-position.

Fig. 5 is a diagrammatic illustration of the construction of pG117ompPRX, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide represents a region encoding an amino acid sequence from glutamine at the 128-position to arginine at the 153-position; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 6 is a diagrammatic illustration showing the structure of a fusion protein PRX encoded by pG117ompPRX, wherein numerical symbols show the amino acid numbers counting from the N-terminus of the fusion protein PRX. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a human glucagon-like peptide-1; Pre GLP-1[G] represents a target peptide comprising an amino acid sequence from the 141- to 184-positions containing GLP-1[G]; and Linker peptide represents an amino acid sequence from glutamine at the 128-position to arginine at the 153-position. A site (arginine 140-X141) corresponding to the OmpT protease cleavage site in the fusion protein PR is shown in this figure.

Fig. 7 is a diagrammatic illustration of the construction of pOmpTTc, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Ap^r

represents an ampicillin-resistance gene; Linker peptide represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHGSGSPSRHPR; OmpT represents OmpT protease gene; lac PO represents *E. coli* lactose promoter operator gene; and trpP represents *E. coli* tryptophan promoter gene.

The nucleotide sequence from the transcription initiation site to the codon of the fifth amino acid following the OmpT protease translation initiation is

5'AATTGTGAGCGGATAACAATTTCACACAGGAAGAATTCATGCGGGCGAAACTT3'

(SEQ ID NO:65)

wherein the underlined part corresponds to the EcoRI recognition site.

With respect to pGP501, see K. Sugimura, Biochem. Biophys. Res. Commun. 153: 753-759, 1988.

Fig. 8 is a diagrammatic illustration of the construction of pOmpTTcB, wherein OmpT represents OmpT protease gene; Tc^r represents a tetracycline-resistance gene; and lac PO represents *E. coli* lactose promoter operator gene.

The nucleotide sequence from the transcription initiation site to the codon of the fifth amino acid following the OmpT protease translation initiation is

5'AATTGTGAGCGGATAACAATTTCACACAGGAAGAATTCAAAATGCGGGCGAAACTG3'

(SEQ ID NO:66)

wherein the underlined part corresponds to the EcoRI recognition site.

Fig. 9 is a diagrammatic illustration of the construction of pOmpTTcC, wherein OmpT represents OmpT protease gene; Tc^r represents a tetracycline-resistance gene; and lac PO represents *E. coli* lactose promoter operator gene.

The nucleotide sequence from the transcription initiation site to the codon of the fifth amino acid following the OmpT protease translation initiation is

5'AATTGTGAGCGGATAAAAATTACAGACAGGAAGAATTCATGCGGGCGAAACTT3'

(SEQ ID NO:67)

wherein the underlined part corresponds to the EcoRI recognition site.

Fig. 10 is a diagrammatic illustration of the construction of pOmpTTcE, wherein OmpT represents OmpT protease gene; Tc^r represents a tetracycline-resistance gene; and lac PO represents *E. coli* lactose promoter operator gene.

The nucleotide sequence from the transcription initiation site to the codon of the fifth amino acid following the OmpT protease translation initiation is

5'AATTGTGAGCGGATAAAAATTACAGACAGGAAGAATTCAAAATGCGGGCGAAACTG3'

(SEQ ID NO:68)

wherein the underlined part corresponds to the EcoRI recognition site.

Fig. 11 is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion protein PRX by OmpT protease.

In this figure, Mr represents molecular weight marker proteins; O represents the lane for purified OmpT protease; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.

A : PRA, V : PRV, L : PRL, I : PRI, P : PRP, F : PRF, W : PRW, M : PRM, G : PRG, S : PRS,
T : PRT, C : PRC, Y : PRY, N : PRN, Q : PRQ, D : PRD, E : PRE, K : PRK, R : PRR, H : PRH.

The 4.9kDa peptide fragment means a peptide fragment containing GLP-1[G] which has been excised by OmpT protease.

Fig. 12 is a diagrammatic illustration of the construction of pG117ompPKX, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-

1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide represents a region encoding an amino acid sequence from glutamine at the 128-position to arginine at the 153-position; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 13 is a diagrammatic illustration showing the structure of a fusion protein PKX encoded by pG117ompPKX, wherein numerical symbols show the amino acid numbers counting from the N-terminus of the fusion protein PKX. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a human glucagon-like peptide-1; Pre GLP-1[G] represents a target peptide comprising an amino acid sequence from the 141- to 184-positions containing GLP-1[G]; and Linker peptide represents the amino acid sequence from glutamine at the 128-position to arginine at the 153-position. A site (lysine 140-X141) corresponding to the OmpT protease cleavage site in the fusion protein PRR is shown in this figure.

Fig. 14 is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion protein PKX by OmpT protease. In this figure, Mr represents molecular weight marker proteins; O represents purified OmpT protease; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.

KA, KS, KK, KR, KD and KE represent respectively PKA, PKS, PKK, PKR, PKD and PKE.

The 4.9kDa peptide fragment means a peptide fragment containing GLP-1[G] which has been excised by OmpT protease.

Fig. 15 is a diagrammatic illustration of the construction of pG117ompPRhANP, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; α-hANP represents a region encoding α-type human atrial natriuretic peptide; Tc^r represents a tetracycline-resistance gene; Linker peptide 1 represents a region encoding an amino acid sequence QFK (SEQ ID NO:69); linker peptide 2 represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHGSGSPYRHPR (SEQ ID NO:70); Linker peptide 3 represents a region encoding an amino acid sequence QMHGYDAELRLYR (SEQ ID NO:71); and lac PO represents *E. coli* lactose promoter operator gene. With respect to pGH α97SII, see "Daichokin o shukushu toshita seirikassei peputido seisankei ni kansuru kenkyu (Study on Physiologically Active Peptide Production System with the Use of *E. coli* as Host)", Koji Magota, Doctoral Dissertation, Kyushu University, 1991.

Fig. 16 is a diagrammatic illustration showing the structure of a fusion protein PRhANP encoded by pG117ompPRhANP, wherein numerical symbols show the amino acid numbers counting from the N-terminus of the fusion protein PRhANP. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; α-hANP represents an α-human atrial natriuretic peptide; and Linker peptide represents the amino acid sequence from glutamine at the 128-position to arginine at the 140-position. A site (arginine 140-serine 141) corresponding to the OmpT protease cleavage site in the fusion protein PRR is shown in this figure.

Fig. 17 is a diagrammatic illustration of the construction of pG117ompPRhCT, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; β-gal197S4D represents a region encoding a protective protein originating in 97 amino acids from the N-terminus of *E. coli* β- galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide 1 represents a region encoding an amino acid sequence EFRHHRRHRLE (SEQ ID NO:72); Linker peptide 2 represents a region encoding an amino acid sequence QMHGYDAELRLYRRHHGSGSPYRHPR; Linker peptide 3 represents a region encoding an amino acid sequence QMHGYDAELRLYR; and lac PO represents *E. coli* lactose promoter operator gene. With respect to pG97S4DhCT[G]R4, see Yabuta, M., Suzuki, Y. and Olisuye, K. Appl. Microbiol. Biotechnol.42: 703-708, 1995.

Fig. 18 is a diagrammatic illustration showing the structure of the fusion protein PRhCT encoded by pG117ompPRhCT, wherein numerical symbols show the amino acid numbers counting from the N-terminus of the fusion protein PRhCT. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; hCT[G] represents a human calcitonin precursor; and Linker peptide represents the amino acid sequence from glutamine at the 128-position to arginine at the 140-position. A site (arginine 140-cysteine 141) corresponding to the OmpT protease cleavage site in the fusion protein PRR is shown in this figure.

Fig. 19 is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion proteins PRhANP and PRhCT by OmpT protease. In this figure, Mr represents molecular weight marker proteins; O represents purified OmpT protease; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.

hANP and hCT represent respectively PRhANP and PRhCT.

Fig. 20 is a diagrammatic illustration of the construction of pGRShANP, wherein β-gal197S represents a region encoding a protective protein derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase; α-hANP represents a region encoding α-type human atrial natriuretic peptide; Tc^r represents a tetracycline-resistance gene; Linker peptide 1 represents a region encoding an amino acid sequence QFK; Linker peptide 2 represents a region encoding an amino acid sequence QFR (SEQ ID NO:73); and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 21 is a diagrammatic illustration showing the whole amino acid sequence of a fusion protein RShANP encoded by pGRShANP wherein the underlined part represents the amino acid sequence of α-hANP (α-type human atrial natriuretic peptide); the double-underlined part represents serine having been converted into another amino acid; and the arrow shows the cleavage site by OmpT protease. The numerical symbols show the amino acid numbers counting from the N-terminus. The protective protein (β-gal97S) derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase comprises the amino acid sequence from methionine at the 1-position to alanine at the 98-position. The linker peptide comprises the amino acid sequence from glutamine at the 99-position to arginine at the 101-position.

Fig. 22 is a diagrammatic illustration of the construction of pGRXhANP, wherein β-gal97S represents a region encoding a protective protein derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase; Modified α-hANP represents a region encoding an α-type human atrial natriuretic peptide derivative having substitution of the N-terminal amino acid into arginine, alanine or cysteine; Tc^r represents a tetracycline-resistance gene; Linker peptide represents a region encoding an amino acid sequence QFK; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 23 is a diagrammatic illustration showing the structure of a fusion protein RXhANP, wherein numerical symbols show the amino acid numbers counting from the N-terminus of the fusion protein PRhANP. β-gal97S represents a protective protein derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase; Modified α-hANP represents an α-type human atrial natriuretic peptide derivative having a substitution of the N-terminal amino acid into arginine, alanine or cysteine; and Linker peptide represents the amino acid sequence from glutamine at the 99-position to arginine at the 101-position. A site (arginine 101-X102) corresponding to the OmpT protease cleavage site in the fusion protein RShANP is shown in this figure.

Fig. 24 is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion proteins RShANP and RXhANP by OmpT protease.

In this figure, Mr represents molecular weight marker proteins; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease. RS, RR, RA and RC respectively represent RShANP, RRhANP, RAhANP and RChANP.

Fig. 25 is a diagrammatic illustration showing the structure of a fusion protein PRRXA encoded by pG117ompPRRXA, wherein -10, -5, -1, +1 and +4 respectively show the -10, -5, -1, +1 and +4-positions concerning the OmpT protease cleavage site of the fusion protein PRR. The amino acid sequence (from -10- to +4-positions) of the fusion proteins PRR and PRRXA are given in the figure. β-gal117S4H represents a protective protein originating in the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a human glucagon-like peptide-1; and Linker peptide corresponds to the amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6. The OmpT protease cleavage site in the fusion protein PRR is shown in this figure. The fusion proteins are represented in bold figures and the substituted alanine is underlined.

Fig. 26 is a diagrammatic illustration of the construction of pG117ompPRR-2A, -3A and -4A. β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1: Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding an amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 27 is a diagrammatic illustration of the construction of pG117ompPRR-5A and -6A, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding an amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 28 is a diagrammatic illustration of the construction of pG117ompPRR-8A, -9A and -10A, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding the amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 29 is a diagrammatic illustration of the construction of pG117ompPRR-1A, wherein β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding the amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose pro-

moter operator gene.

Fig. 30 is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion proteins PRR and PRRXA by OmpT protease. In this figure, Mr represents a protein molecular weight marker; O represents purified OmpT protease; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.
-1A: PRR-1A, -2A: PRR-2A, -3A: PRR-3A, -4A: PRR-4A, -5A: PRR-5A
-6A: PRR-6A, -8A: PRR-8A, -9A: PRR-9A, -10A: PRR-10A.

The 4.9kDa peptide fragment means a peptide fragment containing GLP-1[G] which has been excised by OmpT protease.

Fig. 31 is a diagrammatic illustration showing the structure of the fusion protein PRR-4X encoded by pG117ompPRR-4X, wherein -10, -5, -1, +1 and +4 respectively show the -10, -5, -1, +1 and +4-positions concerning the OmpT protease cleavage site of the fusion protein PRR. The amino acid sequence (from -10- to +4-positions) of the fusion proteins PRR and the substituted amino acid at the -4-position of the fusion protein PRR-4X are given in the figure. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a human glucagon-like peptide-1; and Linker peptide corresponds to an amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6. The OmpT protease cleavage site in the fusion protein PRR is shown in this figure. The fusion proteins are expressed in bold figures.

Fig. 32 is a diagrammatic illustration showing the structure of the fusion protein PRR-6X encoded by pG117ompPRR-6X, wherein -10, -5, -1, +1 and +4 respectively show the -10, -5, -1, +1 and +4-positions concerning the OmpT protease cleavage site of the fusion protein PRR. The amino acid sequence (from -10- to +4-positions) of the fusion proteins PRR and the substituted amino acid at the -6-position of the fusion protein PRR-6X are given in the figure. β-gal117S4H represents a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a human glucagon-like peptide-1; and Linker peptide corresponds to an amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6. The OmpT protease cleavage site in the fusion protein PRR is shown in this figure. The fusion proteins are represented in bold figures.

Fig. 33 is a diagrammatic illustration of the construction of pG117ompPRR-4X (wherein X is K, D, E, N or Q). In this figure, β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding the amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose promoter operator gene.

Fig. 34 is a diagrammatic illustration of the construction of pG117ompPRR-6X (wherein X is K, D, E, N or Q). In this figure, β-gal117S4H represents a region encoding a protective protein derived from the N-terminal 117 amino acids of *E. coli* β-galactosidase; GLP-1[G] represents a region encoding human glucagon-like peptide-1; Tc^r represents a tetracycline-resistance gene; Linker peptide corresponds to the region encoding the amino acid sequence from glutamine at the 128-position to arginine at the 153-position in the amino acid sequence shown in Fig. 6; and lac PO represents *E. coli* lactose promoter operator gene.

In Fig. 35, A is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion proteins PRR and PRR-4X by OmpT protease. In this figure, Mr represents a protein molecular weight marker; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.
-4K: PRR-4K, -4A: PRR-4A, -4N: PRR-4N, -4Q: PRR-4Q, -4D: PRR-4D, -4E: PRR-4E

The 4.9kDa peptide fragment means a peptide fragment containing GLP-1[G] which has been excised by OmpT protease.

In Fig. 35, B is an SDS-PAGE (16%) photograph relating to the cleavage of the fusion proteins PRR and PRR-4X by OmpT protease. In this figure, Mr represents a protein molecular weight marker; - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease.
-6R: PRR-6R, -6K: PRR-6K, -6A: PRR-6A, -6N: PRR-6N, -6Q: PRR-6Q, -6D: PRR-6D.

The 4.9kDa peptide fragment means a peptide fragment containing GLP-1[G] which has been excised by OmpT protease.

Fig. 36 is a diagrammatic illustration showing the structure of the fusion protein RShANPR encoded by pRShANPR, wherein -10, -5, -1, +1 and +4 respectively show the -10, -5, -1, +1 and +4-positions concerning the OmpT protease cleavage site of the fusion protein RShANP. The amino acid sequences (from -10- to +4-positions) of the fusion proteins RShANP and RShANPR are shown in the figure. β-gal97S4H represents a protective protein derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase; α-hANP represents an α-type human atrial natriuretic peptide; and Linker peptide represents the amino acid sequence from glutamine at the 99-position to arginine at the 101-position in the amino acid sequence shown in Fig. 23. The OmpT protease cleavage site in the fusion protein RShANP is shown in this figure. The fusion proteins are expressed in bold figures and the substituted

arginines at the -6- and -4-positions are underlined.

Fig. 37 is a diagrammatic illustration of the construction of pGRShANPR, wherein β-gal97S4H represents a region encoding a protective protein derived from the N-terminal 97 amino acids of *E. coli* β-galactosidase; α-hANP represents a region encoding α-type human atrial natriuretic peptide; Tc^r represents a tetracycline-resistance gene; Linker peptide represents a region encoding an amino acid sequence QFR (SEQ ID NO:73); and lac PO represents *E. coli* lactose promoter operator gene.

DETAILED DESCRIPTION OF THE INVENTION

[0017] Under these circumstances, the present inventors have discovered new substrate specificity profiles by examining amino acid sequences at cleavage sites and in the vicinity with the use of known cleavage sites from the viewpoint that the amino acid sequences around the cleavage sites are important in the substrate recognition and cleavage by OmpT protease. To apply these new substrate specificity profiles to the cleavage of fusion proteins, the inventors have conducted intensive studies and consequently completed the present invention. More specifically, in the method according to the present invention, use is made of the properties of OmpT protease which highly specifically acts so as to cleave exclusively an arginine-X bond or a lysine-X bond (wherein X is an amino acid other than glutamic acid, aspartic acid or proline) existing in specific amino acid sequences including known cleavage sites and the cleavage efficiency is affected by the charges on the amino acids at the -6- and -4-positions of the cleavage site.

[0018] Accordingly, the present invention provides a method of controlling cleavage of a polypeptide by OmpT protease using the properties as described above, which comprises converting the amino acid(s) of a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of said site in said polypeptide into another or other amino acid(s), characterized by (1) setting lysine or arginine as the amino acid at the -1-position of said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position from said site; so that a desired part of said polypeptide is cleavable by OmpT protease and/or an undesired part of said polypeptide is not cleavable by ompT protease.

[0019] In one aspect, therefore, the present invention provides a method of controlling a target polypeptide by OmpT protease which comprises setting lysine or arginine as the amino acid at the -1-position of a cleavage site in an amino acid sequence of a fusion protein containing the target polypeptide, which is cleavable by OmpT protease, and setting an amino acid X (wherein X is an amino acid other than glutamic acid, aspartic acid or proline) as the amino acid at the +1-position of the corresponding amino acid sequence (hereinafter referred to as the corresponding sequence). In another aspect, the present invention provides a method of increasing the cleavage efficiency by excising the target polypeptide while setting amino acid(s) other than acidic amino acid(s) (preferably basic amino acid(s) and still preferably lysine or arginine) as the amino acid(s) at the -6- and/or -4-positions of the cleavage site of the amino acid sequence cleavable by OmpT protease.

[0020] When a fusion protein is expressed with the use of genetic engineering techniques and then cleaved by the method according to the present invention, for example, the fusion protein is expressed in such a manner as to contain "the corresponding sequence" at least as a part of the fusion protein and then the latter is treated by OmpT protease. Thus, the target polypeptide can be released. According to the present invention, moreover, the target polypeptide can be more efficiently excised by setting basic amino acids as the amino acids at the -6- and -4-positions of the cleavage site. The term "target polypeptide" as used herein means any polypeptide to be expressed as a fusion protein or a polypeptide obtained by secretory expression, direct expression, or the like. In a case of fusion proteins to be cleaved by OmpT protease, for example, use may be made of polypeptides which exert physiological activity immediately after the cleavage by OmpT protease or as a result of post-translation modification. Alternatively, production intermediates from which physiologically active peptides are formed after further cleavage following the above-described reaction (i.e., so called precursor peptides) may be employed therefor.

[0021] It is considered that the amino acid sequence to be cleaved by OmpT protease is defined as the sequence of from about -20- to +20-positions relative to the cleavage site. Accordingly, the "corresponding sequence" as used herein may be selected from among the sequence ranging from about -20- to +20-positions relative to the cleavage site of amino acid sequences which has been already known or experimentally confirmed as cleavable by OmpT protease. For example, the "corresponding sequence" may be selected from -20- to -1- positions, from -20- to +1-positions, from -1- to +20-positions or from +1- to +20-positions.

[0022] When an amino acid sequence cleavable by OmpT protease exists, the cleavage can be prevented in the method of the present invention by converting the amino acid at the +1-position of the cleavage site into glutamic acid, aspartic acid or proline. When such a conversion of the amino acid at the +1-position is impossible, the cleavage efficiency can be lowered by converting one or both of the amino acids at the -6- and -4-positions into acidic amino acid(s).

[0023] By combining the above methods, the cleavage by OmpT protease can be controlled.

[0024] This is particularly convenient in a case wherein a fusion protein containing a target polypeptide is produced in *E. coli* employed as a host and then the target polypeptide is excised from the fusion protein with the use of OmpT

protease inherently possessed by *E. coli*.

[0025] Accordingly, the present invention relates to the following methods.

a) A method of controlling cleavage of a polypeptide by OmpT protease which comprises converting amino acid(s) of a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of said site in said polypeptide into other amino acid(s), characterized by (1) setting lysine or arginine as the amino acid at the -1-position relative to said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that a desired part of said polypeptide is cleaved by OmpT protease and/or an undesired part of said polypeptide is not cleaved by ompT protease.

b) The method as described in the above a) of controlling cleavage of polypeptides by OmpT protease which comprises converting amino acid(s) of a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of said site in said polypeptide into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position relative to said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that a desired part of said polypeptide is cleaved by OmpT protease.

c) The method as described in the above b) characterized by (1) setting an amino acid other than glutamic acid, aspartic acid or proline as the amino acid at the +1-position; and/or (2) setting amino acid(s) (preferably basic amino acids and still preferably lysine or arginine) other than acidic amino acids as the amino acid(s) at the -4-position and/or the -6-position relative to said site.

d) A method as described in any of the above a) to a) characterized by, in a case where the amino acid at the -1-position of the sequence site consisting of two arbitrary consecutive amino acids in said polypeptide is neither lysine nor arginine, converting said amino acid into lysine or arginine and setting an amino acid X (wherein X is an amino acid other than glutamic acid, aspartic acid, proline, arginine, lysine, alanine, methionine or valine) as the amino acid at the +1-position so that a desired part of in said polypeptide is cleaved by OmpT protease.

e) The method as described in the above a) for controlling cleavage of polypeptides by OmpT protease which comprises converting amino acid(s) of a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of said site in said polypeptide into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position relative to said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that a undesired part in said polypeptide is not cleaved by OmpT protease.

f) The method as described in the above e) characterized by (1) setting glutamic acid, aspartic acid or proline as the amino acid at the +1-position; and/or (2) setting acidic amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position.

g) A method of applying the method as described in the above e) or f) to a case wherein a gene encoding a polypeptide is expressed in host cells and said polypeptide is otherwise cleaved by OmpT protease at an undesired part.

h) A method of producing a polypeptide by expressing a gene encoding said polypeptide in host cells, characterized by converting amino acid(s) as described in the above a) to g) in a case wherein said polypeptide is otherwise cleaved by OmpT protease at an undesired part.

i) A method as described in any of the above a) to f) which comprises expressing in host cells a gene encoding a fusion protein consisting of a target polypeptide fused with a protective peptide via a cleavage site (optionally located in a linker peptide) and being cleavable by OmpT protease at said cleavage site, and cleaving off the protein at said cleavage site by OmpT protease to thereby obtain the target polypeptide from the fusion protein.

j) The method as described in the above i) wherein an amino acid sequence cleavable by OmpT protease exists in the amino acid sequences of the protective peptide, the linker peptide and/or the target polypeptide constituting said fusion protein.

k) A method of producing a target polypeptide which comprises expressing in host cells a gene, which encodes a fusion protein consisting of a target polypeptide fused with a protective peptide via a cleavage site (optionally located in a linker peptide) and being cleavable by OmpT protease at said cleavage site, and cleaving off the protein at said cleavage site by OmpT protease to thereby obtain the target polypeptide from said fusion protein, characterized by using a method as described in any of the above a) to f) in converting the amino acids at the cleavage site and/or in the vicinity thereof.

l) The method as described in the above k) wherein an amino acid sequence cleavable by OmpT protease exists in the amino acid sequences of the protective peptide, the linker peptide and/or the target polypeptide constituting said fusion protein.

m) A method as described in any of the above g) to l) wherein the host cells is *E. coli*.

n) A method as described in any of the above g) to m) wherein the target polypeptide is a natriuretic peptide.

[0026] Proteins and peptides to which the method according to the present invention is applicable are as follows: Adrenocorticotropic Hormone, Adrenomedullin, Amylin, Angiotensin I, Angiotensin II, Angiotensin III, A-type Natriuretic Peptide, B-type Natriuretic Peptide, Bradykinin, Calcitonin, Calcitonin Gene Related Peptide, Cholecystokinin, Corticotropin Releasing Factor, Cortistatin, C-type Natriuretic Peptide, α-Defesin 1, β-Defesin 1, β-Defesin 2, Delta Sleep-Inducing Peptide, Dynorphin A, Elafin, α-Endorphin, β-Endorphin, γ-Endorphin, Endothelin-1, Endothelin-2, Endothelin-3, Big Endothelin-1, Big Endothelin-2, Big Endothelin-3, Enkephalin, Galanin, Big Gastrin, Gastrin, Gastric Inhibitory Polypeptide, Gastrin Releasing Peptide, Ghrelin, Glucagon, Glucagon-like Peptide 1, Glucagon-like Peptide 2, Growth Hormone Releasing Factor, Growth Hormone, Guanylin, Uroguanylin, Histatin 5, Insulin, Joining Peptide, Luteinizing Hormone Releasing Hormone, Melanocyte Stimulating Hormone, Midkine, Motilin, Neurokinin A, Neurokinin B, Neuromedin B, Neuromedin C, Neuropeptide Y, Neurotensin, Oxytocin, Proadrenomedullin N-terminal 20 Peptide, Parathyroid Hormone, Parathyroid Hormone-Related Protein, Pituitary Adenylate Cyclase Activating Polypeptide 38, Platelet Factor -4, Peptide T, Secretin, Serum Thymic Factor, Somatostatin, Substance P, Thyrotropin Releasing Hormone, Urocortin, Vasoactive Intestinal Peptide, Vasopressin and the like and derivatives thereof (in the case of ANP among the above peptides, for example, use can be made of not only natural ANP consisting of 28 amino acids (i.e., ANP(1-28)) but also derivatives with deletion of amino acids in the amino acid sequence such as ANP(3-28) and ANP(4-28)).

[0027] The present invention will be described in greater detail.

[0028] pG117S4HompRHPR is an expression plasmid which expresses a fusion protein (PR) containing a glucagon-like peptide-1 (GLP-1[G]). The protective protein of this fusion protein consists of a protective protein originating in 117 amino acids from the N-terminus of *E. coli* β-galactosidase, a linker sequence consisting of 35 amino acids including an arginine-arginine sequence, and human glucagon-like peptide-1 (GLP-1[G]). The present inventors have already found out that *E. coli* OmpT protease cleaves the central peptide bond in the arginine-arginine sequence in the linker sequence so as to release the target peptide consisting of 44 amino acids containing GLP-1[G]. The present inventors converted the arginine-arginine sequence of the fusion protein encoded by pG117S4HompRHPR into arginine-X (wherein X represents the amino acid at the +1-position of the cleavage site) by site-specific mutagenesis based on PCR and examined whether or not the thus substituted fusion protein PRX (wherein X represents one letter code of the amino acid (selected from 20 amino acids in total); for example, a fusion protein having a substitution into alanine is represented as PRA) was cleaved by OmpT protease at this site. To express each fusion protein, use was made of an OmpT protease-deficient *E. coli* strain W3110 M25. Since such a fusion protein was accumulated as inclusion body in cells, the cells were disrupted and the inclusion body was collected by centrifugation. Then the inclusion body was solubilized with urea and employed in the OmpT protease reaction. The reaction was carried out by adding 20 mU of OmpT protease to a reaction solution containing 4 M of urea, 50 mM of sodium phosphate (pH 7.0), 2 mM of EDTA and each fusion protein inclusion body. The cleavage of the fusion protein was analyzed by SDS-PAGE (16%) and the N-terminal amino acid sequence of the target peptide thus excised was determined by using a protein sequencer.

[0029] As a result, it was clarified for the first time by the present inventors that OmpT protease has the activity of cleaving the center peptide bond in the arginine-X sequence wherein X is an amino acid other than aspartic acid, glutamic acid or proline. That is to say, the present inventors clarified that OmpT protease has the activity of cleaving not only the amino acid sequences reported so far (namely, arginine-arginine, arginine-lysine, lysine-arginine, lysine-lysine, arginine-alanine, arginine-methionine and arginine-valine) but also the amino acid sequences represented by arginine-X (wherein X is an amino acid other than aspartic acid, glutamic acid and proline).

[0030] By using these fusion proteins, it was further examined whether or not the lysine-X (wherein X is located at the +1-position of the cleavage site and represents alanine, serine, lysine, arginine, aspartic acid or glutamic acid) was cleaved and similar results were obtained thereby. It is anticipated that OmpT protease is largely affected by the amino acid sequence in the vicinity of the cleavage site. Therefore, the examination was further carried out to study whether or not fusion proteins PRhANP and PRhCT (wherein the target peptide region of the fusion protein PR was substituted respectively with α-hANP (α-type human atrial natriuretic peptide) and hCT[G] (human calcitonin precursor)) could be cleaved by OmpT protease. As a result, it was found out that PRhANP was cleaved by OmpT protease between arginine and serine and thus α-hANP was excised therefrom. On the other hand, PRhCT was not cleaved by OmpT protease. The fact that the arginine-cysteine sequence of PRhCT was not cleaved by OmpT protease indicates that the recognition and cleavage of a substrate by OmpT protease are affected by the amino acid sequence in the vicinity of the cleavage site, which supports the significance of using known amino acid sequences cleaved by OmpT protease as proposed by the present inventors.

[0031] The above-described results were obtained by a series of studies with the use of the fusion protein PR having amino acid sequences of known cleavage sites. Moreover, it was examined whether or not similar results would be obtained by substituting the amino acid at the +1-position of another fusion protein RShANP (i.e., an α-hANP fusion protein having an amino acid sequence different from PR around the cleavage site). The fusion protein RShANP employed in this examination consists of β-gal197S, which originates in 97 amino acids from the N-terminus of *E. coli* β-galactosidase, as a protective protein, and α-hANP bonded thereto via a linker consisting of three amino acids (glutamine-phenylalanine-arginine). Attempts were made to cleave fusion proteins by OmpT protease wherein the

amino acid at the +1-position of this fusion protein had been substituted by arginine, alanine and cysteine. As a result, it was found out that these fusion proteins having been substituted the amino acid at the +1-position were also cleaved by OmpT protease to give the N-terminal derivative of α-hANP.

[0032] These results indicate that when a region having a known OmpT protease-cleavage sequence (wherein the -1- and +1-positions at the cleavage site are represented by Arginine-X or lysine-X) is employed and said X is substituted by an amino acid other than aspartic acid, glutamic acid or proline, the fusion protein thus substituted is still cleaved by OmpT protease. Therefore, when a fusion protein consisting of a protective peptide, the amino acid sequence of an OmpT protease-cleavage site and a target peptide in this order is to be cleaved by this enzyme, it is possible to select the amino acid added to the N-terminus of the target peptide from among amino acids other than aspartic acid, glutamic acid and proline. By using this method, a derivative having a different amino acid at the N-terminus of a target peptide can be constructed. It is also possible to substitute the N-terminal amino acid so as to increase the separation/purification efficiency. It is also possible to convert a sequence which is not cleaved to a cleavable sequence.

[0033] By using the results that a peptide having X as aspartic acid, glutamic acid or proline is not cleavable by OmpT protease, moreover, it is possible to convert a fusion protein or protein into one which cannot be digested by OmpT protease. More specifically, it is sometimes observed that, in the process of producing a protein expressed in *E. coli*, the target protein can be hardly isolated due to digestion by OmpT protease. In such a case, the protein can be converted into a fusion protein or protein by substituting the recognition amino acid at the +1-position of the cleavage site into aspartic acid, glutamic acid or proline, which obviously facilitates the production.

[0034] The present inventors further substituted amino acids at the -10- to -1-positions of the OmpT protease cleavage site of the fusion protein PRR and examined the cleavage of these fusion proteins by OmpT protease. As a result, they have found that the N-terminal amino acid sequence of the cleavage site affected the cleavage efficiency. In particular, the cleavage efficiency was elevated by setting a basic amino acid such as arginine or lysine as the amino acid at the -4-position but lowered by setting an acidic amino acid such as aspartic acid or glutamic acid. Similar results were obtained concerning the amino acid at the -6-position. Based on these results, it is considered that OmpT protease recognizes the electric charges of the amino acids at these positions. When arginine at the -1-position was substituted by alanine, no cleavage occurred. This fact indicates again that the amino acid at this position serves an important role in the cleavage.

[0035] In addition, RShANP which is an α-hANP fusion protein having a different amino acid sequence around the cleavage site form PRR was used, and the increase in the cleavage efficiency was observed in a fusion protein RShANPR wherein tyrosine and alanine at the -6- and -4-positions respectively in the cleavage site had been substituted both by arginine.

[0036] Accordingly, the cleavage efficiency can be improved by converting either or both of the amino acids at the -6- and -4-positions into a basic amino acid, while the cleavage efficiency can be lowered by converting these amino acids into an acidic one. That is to say, the cleavage efficiency can be controlled to a certain extent without substituting the amino acids at the cleavage site (i.e., -1- or +1-position).

[0037] Moreover, experimental operations not described in the Examples will be first described in detail.

(1) Materials and methods

[0038] Unless otherwise stated in the Examples, the experimental operations were carried out as follows.

[0039] Synthesis of DNA primers was entrusted to Pharmacia. Nucleotide sequences were determined by using a A.L.F. DNA Sequencer (manufactured by Pharmacia) with the use of a Thermo Sequenase florescent labeled primer cycle sequencing kit with 7-deaze dGTP (manufactured by Amersham). Plasmid DNAs were isolated from *E. coli* by using a PI-100Σ (manufactured by Kurabo). To cleave DNA with restriction enzymes, the reaction was carried out at 500 to 2000 U/ml for 2 hours. The structure of a plasmid was analyzed in 10 μl of a liquid reaction mixture with the use of 0.5 to 1 μg of DNA. A DNA fragment was prepared in 30 μl of a liquid reaction mixture with the use of 5 to 10 μg of DNA. The reaction conditions (temperature, buffer, etc.) were determined according to the manufacturer's instructions. Samples for agarose gel electrophoresis were prepared by adding a 1/10 volume of sample buffer to the liquid reaction mixture. As the buffer for agarose gel electrophoresis, use was made of TAE buffer (40 mM Tris-acetic acid, 1 mM EDTA). The electrophoresis was effected at 100 V for 30 minutes to 1 hour. After staining with an aqueous ethidium bromide solution, the gel was UV-irradiated to detect DNA bands. The concentration of the agarose gel was adjusted to 0.8 or 2.0%(w/v) depending on the size of the DNA fragment to be fractionated. After the agarose gel electrophoresis, the target DNA band was cut out and the DNA was extracted from the gel by using SUPREC-01 (manufactured by Takara Shuzo). This DNA solution was treated with phenol/chloroform, then precipitated from ethanol and dissolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Ligation reaction was carried out by using Ligation high (manufactured by Toyobo) at a predetermined reaction mixture composition at 16°C for 30 minutes or overnight. PCR was carried out by using KOD Dash or KOD DNA polymerase (manufactured by Toyobo). The PCR conditions (temperature, buffer, etc.)

and the composition of the liquid reaction mixture were determined each according to the manufacturer's instructions.

[0040] Transformation of *E. coli* with a plasmid was carried out by the calcium chloride method by using JM109 strain purchased from Takara Shuzo as competent cells. The transformant was selected with the use of tetracycline (10 μg/ml). Unless otherwise stated in Examples, JM109 was employed for the transformation of *E. coli*.

(2) Measurement of enzymatic activity of OmpT protease

[0041] Activity of OmpT protease was measured by using Dynorphine A as a substrate (manufactured by Peptide Institute Inc.).

[0042] A 5 μg aliquot of 1 mg/ml Dynorphine A was added to 40 μl of 50 mM sodium phosphate (pH 6.0) containing 0.1% Triton X-100. Then 5 μl of a sample for measuring OmpT protease activity was added thereto and the reaction was initiated. The reaction was carried out at 25°C for 10 minutes and then stopped by adding 5 μl of 1 N HCl. The liquid reaction mixture was centrifuged (10000 x g, 2 minutes). The supernatant was collected and a 20 μl portion thereof was analyzed by HPLC. The HPLC analysis was carried out by using YMC PROTEIN RP column at column temperature of 40°C and at flow rate of 1 ml/min. After washing with 10% acetonitrile containing 0.1% of trifluoroacetic acid for 3 minutes, the mixture was subjected to linear gradient elution with 10-15% acetonitrile containing 0.1% trifluoroacetic acid for 10 minutes. The absorption at 220 nm was monitored and thus the digestion product peptide YGGFLR was detected. The unit of OmpT protease activity was defined as the cleavage of 1 μmol Dynorphine A at 25°C per minute under these conditions.

(3) SDS-polyacrylamide gel electrophoresis

[0043] SDS-polyacrylamide gel electrophoresis was carried out by using 16% Wide-PAGEmini (manufactured by Tefco) as a gel, Tricine electrophoretic buffer (manufactured by Tefco) as an electrophoretic buffer, and molecular weight marker proteins (manufactured by Tefco) as molecular weight markers. The equivalent amount of 2 x SDS-PAGE sample buffer containing 4 M urea (provided that the urea is not contained in a case of analyzing OmpT protease protein) was added to a sample and the mixture was heated to 100°C for 2 minutes. Then 10 μl portion thereof was electrophoresed under the conditions according to Tefco's instructions. After the completion of the electrophoresis, the gel was stained with a staining solution containing Coomassie Brilliant Blue R-250.

(4) Preparation of inclusion body

[0044] Fusion proteins PRX, PKX, PRhANP, PRhCT, RShANP, RXhANP, PRRXA, PRR-4X, PRR-6X and RShANPR were each prepared as an inclusion body in the following manner.

[0045] *E. coli* expressing each of PRX, PKX, PRhANP, PRhCT, RShANP, RXhANP, PRRXA, PRR-4X, PRR-6X and RShANPR was cultured under rotation at 150 rpm, 37°C overnight in 2 l Erlenmeyer flasks containing 400 ml of an LB liquid medium (0.5% (w/v) yeast extract, 1% (w/v) tryptone, 0.5% sodium chloride) containing 10 mg/l tetracycline. On the next day, the cells were collected by centrifugation (4°C, 6000 x g, 10 minutes) and disrupted by ultrasonication. Deionized water was added to this disrupted cell solution to give a total volume of 30 ml. Then the mixture was centrifuged (4°C, 25000 x g, 15 minutes) and the supernatant was discarded. The precipitate fraction (inclusion body) was recovered and further suspended in 30 ml of 50 mM Tris HCl (pH 8.0) containing 5 mM EDTA and 1% Triton X-100. The suspension was centrifuged (4°C, 25000 x g, 15 minutes). The precipitate thus obtained was suspended in deionized water and centrifuged (4°C, 25000 x g, 15 minutes). Then the precipitate was recovered and deionized water was added thereto to give a total volume of 1.5 ml. After suspending the precipitate, the suspension was centrifuged (4°C, 10000 x g, 30 minutes) to give precipitate. Then the above procedure was repeated so as to give a suspension of the precipitate in deionized water of OD_{660} = 100 or OD_{660} = 200. The inclusion bodies thus prepared were employed as substrates in the OmpT protease reaction.

(5) OmpT protease reaction

[0046] By using as the substrate PRX, PKX, PRhANP, PRhCT, PRRXA, PRR-4X and PRR-6X, the OmpT protease reaction was carried out in the following manner. To 20 μl of 10 M urea were added 2.5 μl of 1 M sodium phosphate (pH 7.0) and 2 μl of 50 mM EDTA. Then 10 μl of a fusion protein inclusion body (OD_{660} = 100) was added thereto and the inclusion body was dissolved. After adding 10.5 μl of water, 5 μl of 4 U/ml (20 U/ml in the case of PRR-4X, 1 U/ml in the case of PRR-6X) of OmpT protease was added thereto and the reaction was initiated at a liquid reaction mixture volume of 50 μl. The reaction was carried out at 25°C for 30 or 60 minutes.

[0047] The peptides obtained by the OmpT protease reaction with the use of PRX, PKX, PRRXA, PRR-4X and PRR-6X as the substrates were each isolated and quantitated by HPLC under the conditions as specified below. To the

OmpT protease reaction mixture, the equivalent amount of 12% acetic acid and 4 M urea were added to thereby cease the reaction. Then the liquid reaction mixture was centrifuged (10000 x g, 2 minutes) and 20 µl portion or a 50 µl portion of the supernatant was treated with YMC PROTEIN RP column. HPLC was carried out at column temperature of 40°C at a flow rate of 1 ml/min. After performing linear gradient elution with 30-50% acetonitrile containing 0.1% trifluoroacetic acid for 16 minutes, the absorption at 214 nm was monitored and thus the peptide was isolated and quantitated.

[0048] By using as the substrates RShANP and RXhANP, the OmpT protease reaction was carried out in the following manner. To 20 µl of 10 M urea were added 2.5 µl of 1 M sodium phosphate (pH 7.0) and 2 µl of 50 mM EDTA. Then 5 µl of a fusion protein inclusion body (OD_{660} = 200) was added thereto and the inclusion body was dissolved. After adding 15.5 µl of water, 5 µl of 10 U/ml of OmpT protease was added thereto and the reaction was initiated at 50 µl of a liquid reaction mixture volume. The reaction was carried out at 37°C for 120 minutes.

[0049] By using as the substrates RShANP and RShANPR, the OmpT protease reaction was carried out in the following manner. To 8 µl of 10 M urea were added 1.0 µl of 1 M sodium phosphate (pH 7.0) and 0.8 µl of 50 mM EDTA. Then 4 µl of the fusion protein inclusion body (OD_{660} = 100) was added thereto and the inclusion body was dissolved. After adding 4.2 µl of water, 2 µl of 20 U/ml OmpT protease was added thereto and the reaction was initiated at a liquid reaction mixture volume of 20 µl. The reaction was carried out at 25°C for 90 minutes.

[0050] The peptides obtained by the OmpT protease reaction with the use of PRhANP, RShANP, RXhANP and RShANPR as the substrates were each isolated and quantitated by HPLC under the conditions as specified below. To the OmpT protease reaction mixture, the equivalent amount of 12% acetic acid and 4 M urea were added to thereby cease the reaction. Then the liquid reaction mixture was centrifuged (10000 x g, 2 minutes) and 20 µl portion or 50 µl portion of the supernatant was treated with YMC A-302 ODS column. HPLC was carried out at a column temperature of 40°C and a flow rate of 1 ml/min. After performing linear gradient elution with 21.5-32% acetonitrile containing 0.1% trifluoroacetic acid for 15 minutes, the absorption at 214 nm was monitored and thus the peptide was isolated and quantitated.

(6) Analysis of N-terminal amino acid sequence of peptide

[0051] The N-terminal amino acid sequence of each peptide thus obtained was determined with respect to 5 amino acid residues by using Protein Sequencer 477A-120A or PROCISE 492 (manufactured by ABI).

EXAMPLES

[0052] The present invention will be described in greater detail by reference to the following Examples.

Example 1: Preparation of fusion protein PRX

[0053] OmpT protease is an endoprotease which exists in *E. coli* outer membrane. Although this enzyme has a high substrate specificity, the characteristics of the amino acid sequences in the substrate recognized by the enzyme have not been sufficiently clarified so far. It is known that OmpT protease cleaves the center bond of basic amino acid pairs (arginine-arginine, arginine-lysine, lysine-arginine and lysine-lysine). In addition, it is reported that OmpT protease cleaves the C-terminal peptide bond of basic amino acid (arginine-methionine, arginine-alanine and arginine-valine). However, OmpT protease does not always cleave these sites in the amino acid sequences of proteins and peptides, and the cleavage by this enzyme is greatly affected by the amino acid sequence in the vicinity of the cleavage site. It is therefore estimated that the enzyme has a high substrate specificity and cleaves exclusively specific sites. The present inventors expected that a novel substrate specificity of this enzyme would be found by examining the amino acid sequence at the +1-position of the cleavage site with the use of known cleavage sites of this enzyme. From this viewpoint, they conducted the following experiments.

[0054] At the +1-position of a fusion protein PR (i.e., a fusion protein consisting of a protective protein (β-gal117S4H) derived from the 117 amino acids in the N-terminus of *E. coli* β-galactosidase and human glucagon-like peptide-1 (GLP-1[G])) having the structure as shown in Fig. 4 which is cleavable by OmpT protease, an amino acid substitution was made to thereby form fusion protein PRX (Fig. 6: wherein X represents one letter code of the amino acid substituted (20 types in total); namely, a fusion protein having substitution into alanine is represented as PRA). In the fusion protein PRX, the OmpT protease cleavage site -RLYR↓RHHG- (SEQ ID NO:1)of the original fusion protein PR was converted into -RLYRXHHG- (SEQ ID NO:2). Then cleavage by OmpT protease was examined.

[0055] The fusion protein PRX was prepared by the following five steps.

(1) Step 1: Construction of pG117S4HR6GLP-1 (Fig. 1)

[0056] First, plasmid pG117S4HR6GLP-1 was constructed. This plasmid carried a sequence arginine-arginine

which was inserted as the OmpT protease recognition/cleavage site into the linker moiety of the fusion protein. In the construction, the R6 synthesis DNA sequence (See Fig. 1) was inserted into the StuI site of pG117S4HGP (see Japanese laid-Open Patent Publication No. 9-296000 and EP 794255) to thereby give pG117S4HR6GLP-1. In Fig. 1, β-gal117S4H represents a protective protein derived from the 117 amino acids in the N-terminus of *E. coli* β-galactosidase and GLP-1[G] represents human glucagon-like peptide-1.

(2) Step 2: Construction of pG117S4HompRHKR (Fig. 2)

[0057] To further enhance the cleavage efficiently by OmpT protease, the sequence in the R6 moiety was modified in the following manner. A 3.2 kbp fragment (fragment 1) obtained by cleaving pG117S4HR6GLP-1 by NsiI and HindIII, a 0.2 kbp fragment (fragment 2) obtained by pG117S4HR6GLP-1 by BamHI and HindIII, and an L1 synthesis DNA (see Fig. 2) encoding an amino acid sequence L1 (see Fig. 2) having an arginine-arginine sequence (i.e., the recognition/cleavage site of OmpT protease) were ligated together to thereby construct pG117S4HompRHKR.

(3) Step 3: Construction of pG117S4HompRHPR (Fig. 3)

[0058] Since a lysine-arginine (KR) sequence (corresponding to the 152- and 153-positions in Fig. 4) positioned immediately before the N-terminus of the fusion protein GLP-1[G] expressed by pG117S4HompRHKR is cleavable by OmpT protease, it has been known by preliminarily experiments that this fusion protein is cleaved at two sites when treated with OmpT protease. To facilitate the analysis, therefore, this sequence was substituted by proline-arginine (PR) to thereby to prevent it from cleavage by OmpT protease. Primers P1:5'-GACTCAGATCTTCCTGAGGCCGAT-3' (SEQ ID NO:3) and P2:5'-AAAGGTACCTTCCGCATGCCGCGGATGTCGAGAAGG-3' (SEQ ID NO:4) were synthesized and PCR was performed with the use of pG117S4HompRHKR as a template to give a 0.1 kbp DNA fragment. The obtained fragment was treated with BglII and SphI (fragment 3) and then ligated to a 3.2 kbp fragment (fragment 4) obtained by cleaving pG117S4HompRHKR by BglII and HindIII and a 0.2 kbp fragment (fragment 5) obtained from pG117S4HompRHKR by SphI and HindIII to thereby construct pG117S4HompRHPR. Fig. 4 shows the whole amino acid sequence of the fusion protein PR encoded by pG117S4HompRHPR.

(4) Step 4: Construction of pG117ompPRX (Fig. 5)

[0059] The OmpT protease cleavage site -RLYR↓RHHG- of the fusion protein PR encoded by pG117S4HompRHPR was converted into -RLYRXHHG- (wherein X represents an amino acid selected from the 20 types). This conversion was carried out by introducing a mutation into pG117S4HompRHPR.

[0060] The mutation was introduced by PCR by using pG117S4HompRHPR as a template. As primers, use was made of P3:5'-ACCCCAGGCTTTACACTTTA-3' and P4X:5'-CCGGATCCGTGATGNNNGCGATACAGGCG-3' (wherein X represents one letter code of the amino acid (20 types in total); and NNN represents AGC, AAC, CAG, GAT, CGG, GAA, CCA, CAT, GCC, AGA, GGT, GCA, GTA, GTT, CTG, GTC, TTC, TTT, ACG or ATG when conversion into alanine, valine, leucine, isoleucine, praline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine or histidine is intended respectively). The PCR product thus obtained was digested by PvuI and BamHI to give a 0.3 kb fragment (fragment 6). Furthermore, PCR was carried out by using pG117S4HompRHPR as a template and P5:5'-ACGGATCCGGTTCCCCTTATCGACATCCG-3' and P6:5'-TTGCGCATTCACAGTTCTCC-3' as primers. The PCR product thus obtained was digested by BamHI and HIndIII to give a 0.2 kbp fragment (fragment 7). These fragments 6 and 7 and a 3.0 kbp fragment (fragment 8) obtained by digesting pG117S4HompRHPR by PvuI and HindIII were ligated so as to carry out transformation. Plasmids were isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed so that it was identified as the expression plasmid of the target fusion protein PRX. These plasmids were collectively referred to as pG117ompPRX (wherein X represents one letter code of the amino acid (20 types in total); namely, a fusion protein having the substitution into alanine is expressed by pG117ompPRA) (Fig. 5).

(5) Step 5: Preparation of fusion protein PRX

[0061] When pG117ompPRX is expressed in *E. coli*, the fusion protein PRX (Fig. 6) is expressed as inclusion body. In a case where OmpT protease is expressed in *E. coli*, the inclusion body is cleaved by OmpT protease merely by dissolving with urea. To avoid the cleavage, therefore, pG117ompPRX was transfected into W3110 M25 (i.e., an OmpT protease-deficient *E. coli* strain) and thus the fusion protein PRX was prepared in the form of inclusion body.

Example 2: Preparation of purified OmpT protease specimen

[0062] To prepare purified OmpT protease, the OmpT protease expression plasmid was transfected into an *E. coli* W3110 strain to construct an OmpT protease high-expression *E. coli* strain. From the membrane fraction of this strain, OmpT protease was purified by the following five steps.

(1) Step 1: Construction of pOmpTTc (Fig. 7)

[0063] To enhance the expression level of OmpT protease, an OmpT protease expression plasmid pOmpTTc was constructed. EcoRI- and SalI-restriction enzyme sites were introduced by site-specific mutagenesis respectively into immediately before the translation initiation site and the 3'-end of the OmpT gene in plasmid pGP501 (Sugimura, K. Biochem. Biophys. Res. Commun.153: 753-759, 1988) containing an OmpT protease gene. By digesting the plasmid by these restriction enzymes, a 1.3 kbp fragment (fragment 9) was obtained.

[0064] To introduce an EcoRI restriction enzyme site into the downstream of lac promoter, PCR was carried out by using pG117S4HompRHPR as a template and P7:5'-GCGGGTGTTGGCGGGTGTCG-3' and P8:5'-TGAATTCTTCCT-GTGTGAAATTGTTAT-3' as primers. The PCR product thus obtained was digested by EcoRI and AlwNI to give a 0.5 kbp fragment (fragment 10). These fragments 9 and 10 and a 2.3 kbp fragment (fragment 11) obtained from pG117S4HompRHPR by A1wNI and SalI were ligated to thereby construct pOmpTTc.

(2) Step 2: Construction of pOmpTTcB (Fig. 8)

[0065] In a method for constructing plasmids showing expression of proteins at high level in *E. coli* (Shunji Natori, Yoshinobu Nakanishi, Zoku Iyakuhinn no Kaihatsu (Sequel To Development of Drugs), vol. 7, 29-61, 1991, Hirokawa Shoten), attempts were made to improve pOmpTTc in the following two points. ① To place the OmpT protease translation initiation site 9 bases down stream of the SD sequence. ② To modify nucleotides so as to minimize the formation of stems or loops in the secondary structure of mRNA between the transcription initiation site and the fifth amino acid from the initiation of OmpT protease translation. After constructing pOmpTTcB (Fig. 8) by the improvement ① and oPmpTTcC (Fig. 9) by the improvement ②, pOmpTTcE (Fig. 10) having been subjected to both of the improvements ① and ② was constructed.

[0066] pOmpTTcB with the improvement ① was constructed in the following manner (Fig. 8).

[0067] pOmpTTc was digested by HincII and MfeI to give a 1.0 kbp fragment (fragment 12) and pOmpTTc was digested by EcoRI and MfeI to give a 2.9 kbp fragment (fragment 13).

[0068] To perform the improvement ①, PCR was carried out by using pOmpTTc as a template and P9:5'-TGAAT-TCAAAATGCGGGCGAAACTGCTGGG-3' and P10:5'-TGCCGAGGATGACGATGAGC-3' as primers. The PCR product thus obtained was digested by EcoRI and HincII and the resulted 0.2 kbp fragment (fragment 14) was ligated to the fragments 12 and 13 to thereby construct pOmpTTcB.

(3) Step 3: Construction of pOmpTTcC (Fig. 9)

[0069] pOmpTTcC with the improvement ② was constructed in the following manner.

[0070] pOmpTTc was digested by EcoRI and SalI to give a 1.3 kbp fragment (fragment 15), and by AlwNI and SalI to give another 2.3 kbp fragment (fragment 16).

[0071] To perform the improvement ②, PCR was carried out by using pOmpTTc as a template and P11:5'-CTATCGTCGCCGCACTTATG-3' and P12:5'-TGAATTCTTCCTGTCTGTAATTTTTATCCGCTCACAATT-3' as primers. The PCR product thus obtained was digested by EcoRI and AlwNI. The resulted 0.5 kbp fragment (fragment 17) was ligated to the fragments 15 and 16 to thereby construct pOmpTTcC.

(4) Step 4: Construction of pOmpTTcE (Fig. 10)

[0072] To improve the expression level of OmpT protease, pOmpTTcE with the improvements of ① and ② was constructed in the following manner.

[0073] The 1.3 kbp fragment (fragment 18) obtained by digesting pOmpTTcB by EcoRI and SalI was ligated to a 2.8 kbp fragment (fragment 19) obtained by digesting pOmpTTcC by EcoRI and SalI to thereby construct pOmpTTcE.

(5) Step 5: Preparation of purified OmpT protease specimen

[0074] To obtain a purified OmpT protease specimen, pOmpTTcE was transfected into *E. coli* W3110 strain to thereby give an OmpT protease high expression *E. coli* strain. Next, the OmpT protease high expression *E. coli* strain

was cultured in the following manner and OmpT protease was purified.

[0075] The W3110/pOmpTTcE strain was incubated under rotation at 37°C overnight in a 500 ml Erlenmeyer flask with the use of 100 ml of a liquid LB medium containing 10 mg/l of tetracycline. On the next day, it was transferred into an culture vessel equipped with a stirrer containing a medium 21 containing 4 g/l K_2HPO_4, 4 g/l KH_2PO_4, 2.7 g/l Na_2HPO_4, 0.2 g/l NH_4Cl, 1.2 g/l $(NH_4)_2SO_4$, 4 g/l yeast extract, 2 g/l $MgSO_4 \cdot 7H_2O$, 40 mg/l $CaCl_2 \cdot 2H_2O$, 40 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $MnSO_4 \cdot nH_2O$, 10 mg/l $AlCl_3 \cdot 6H_2O$, 4 mg/l $CoCl_2 \cdot 6H_2O$, 2 mg/l $ZnSO_4 \cdot 7H_2O$, 2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 1 mg g/l $CuCl_2 \cdot 2H_2O$, 0.5 mg/l H_3BO_4, 1 g/l glucose, 10 g/l glycerol and 10 mg/l tetracycline and cultivated therein at 37°C for 12 hours. After the completion of the cultivation, the culture medium was centrifuged (4°C, 6000 x g, 10 minutes) to give 80 g of packed cells. These cells were suspended in 600 ml of 50 mM Tris-HCl (pH 7.5) and the suspension was centrifuged (4°C, 6000 x g, 10 minutes) to thereby collect the cells. After repeating this procedure, the cells were suspended in 600 ml of 50 mM Tris-HCl (pH 7.5) and disrupted with a Manton-Gorlin. The suspension of the disrupted cells was centrifuged (4°C, 1000 x g, 10 minutes) and the precipitate was discarded and the supernatant was recovered. The supernatant was further centrifuged (4°C, 36000 x g, 40 minutes). The precipitate was recovered, suspended in 150 ml of 50 mM Tris-HCl (pH 7.5) and centrifuged again (4°C, 36000 x g, 40 minutes). To a 1/6 portion of the precipitate thus obtained was added 120 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1% salcosyl. After suspending, the mixture was shaken at 10°C for 1 hour. After 1 hour, it was centrifuged (4°C, 36000 x g, 40 minutes) and the precipitate was recovered. Further, it was suspended in 120 ml of 50 mM Tris-HCl containing 0.1% of Triton X-100 and 5 mM of EDTA and shaken at room temperature for 1 hour. Next, it was centrifuged (4°C, 36000 x g, 40 minutes) and the supernatant was recovered to give a crude enzyme specimen.

[0076] 120 ml of this crude enzyme specimen was applied onto Benzamidine Sepharose 6B Column (12 mm in diameter x 70 mm, 8 ml) having been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100 (hereinafter referred to as the buffer A) at a flow rate of 4 ml/min and then washed with 80 ml of the buffer A. Then the column was eluted with the buffer A containing 0.3 M NaCl and the eluate was taken up in 10 ml portions to give 8 fractions in total.

[0077] The fifth fraction, which was proved as being homogeneous by 16% SDS-PAGE, was referred to as a purified OmpT protease specimen. When determined by using a Coomassie Plus Protein Assay Reagent (manufactured by PIERCE) and bovine serum albumin as a standard, the protein concentration of the purified OmpT protease specimen was 120 μg/ml. The OmpT protease activity measured by using Dynorphine A as a substrate was 40 U/ml.

Example 3: Cleavage of PRX by OmpT protease

[0078] It was examined whether or not the fusion protein PRX (Fig. 6), which was constructed by substituting the amino acid at the +1-position (141-position from the N-terminus) of the fusion protein PR having a structure cleavable by OmpT protease (Fig. 4), was cleaved by OmpT protease. PRX was reacted with the purified OmpT protease specimen at pH 7.0 for 30 minutes at 25°C. After the enzymatic reaction, SDS-PAGE analysis was performed. Fig. 11 shows the results wherein - represents a lane free from OmpT protease, and + represents a lane of fusion protein treated with OmpT protease.

[0079] In PRD and PRE, no cleavage by OmpT protease was observed (Fig. 11, lanes D and E). In contrast, cleavage by OmpT protease was observed in the proteins other than PRD and PRE.

[0080] To identify the cleavage site, peptide digestion products obtained after the OmpT protease treatment were isolated by HPLC and the N-terminal amino acid sequences were determined. The OmpT protease cleavage sites thus identified are listed in Table 1.

Table 1

OmpT protease cleavage site of fusion protein PRX	
PRX	Cleavage site
PRA	LYR↓AHHGSG (SEQ ID NO:16)
PRV	LYR↓VHHGSG (SEQ ID NO:17)
PRL	LYR↓LHHGSG (SEQ ID NO:18)
PRI	LYR↓IHHGSG (SEQ ID NO:19)
PRP	LYRPHHGSG (SEQ ID NO:20)
PRF	LYR↓FHHGSG (SEQ ID NO:21)
PRW	LYR↓WHHGSG (SEQ ID NO:22)

Table 1 (continued)

OmpT protease cleavage site of fusion protein PRX	
PRX	Cleavage site
PRM	LYR↓MHHGSG (SEQ ID NO:23)
PRG	LYR↓GHHGSG (SEQ ID NO:24)
PRS	LYR↓SHHGSG (SEQ ID NO:25)
PRT	LYR↓THHGSG (SEQ ID NO:26)
PRC	LYR↓CHHGSG (SEQ ID NO:27)
PRY	LYR↓YHHGSG (SEQ ID NO:28)
PRN	LYR↓NHHGSG (SEQ ID NO:29)
PRQ	LYR↓QHHGSG (SEQ ID NO:30)
PRD	LYRDHHGSG (SEQ ID NO:31)
PRE	LYREHHGSG (SEQ ID NO:32)
PRK	LYR↓KHHGSG (SEQ ID NO:33)
PRR	LYR↓RHHGSG (SEQ ID NO:34)
PRH	LYR↓HHHGSG (SEQ ID NO:35)
↓ stands for an OmpT protease cleavage site. The region from leucine at the 138-position (from the N-terminus) to glycine at the 146-position in the fusion protein PRX is shown as the amino acid sequence at the cleavage site.	

[0081] In PRP, cleavage was observed not at -RX- but exclusively at -ELR↓LYRPHHG-. In all of the proteins other than PRD, PRE and PRP, however, cleavage was observed at R↓X- (Table 1). Based on these results, it is assumed that amino acid sequences having one of the 17 amino acids [namely, those other than aspartic acid and glutamic acid (acidic amino acids) and proline (an imino acid)] at the +1-position are cleavable by OmpT protease.

Example 4: Preparation of fusion protein PKX

[0082] Similarly, to examine whether or not the cleavage by OmpT protease can be performed after substituting the amino acid at the +1-position by one of the amino acids other than aspartic acid and glutamic acid (acidic amino acids) and proline also in the case when the OmpT protease cleavage site has a lysine as the basic amino acid at the -1-position, PKX wherein -RLYRXHHG- of the fusion protein PRX was converted into -RLYKXHHG-, was constructed (Fig. 13). In the following example, X was selected from alanine, serine, lysine, arginine, aspartic acid and glutamic acid (represented in one letter code of each amino acid). Among these amino acids, lysine and arginine, which formed a basic amino acid pair, were employed as a positive control. Alanine and serine were employed because they showed relatively high cleavage efficiency in Example 3. Aspartic acid and glutamic acid were employed to examine whether PKD and PKE were cleavable or not, since PRD and PRE containing these amino acids were not cleavable.

[0083] The fusion protein PKX was prepared by the following two steps.

(1) Step 1: Construction of pG117ompPKX (Fig. 12)

[0084] The plasmid pG117ompPKX (wherein X is A, S, K, R, D or E) (Fig. 12) encoding the fusion protein PKX (wherein X is A, S, K, R, D or E) (Fig. 13) was formed in the following manner.
The conversion of -RLYRXHHG- into -RLYKXHHG- (wherein X is A, S, K, R, D or E) was conducted by PCR.

[0085] PCR was carried out by using pG117ompPRA, pG117ompPRS, pG117ompPRK, pG117ompPRR, pG117ompPRD and pG117ompPRE as templates and P3:5'-ACCCCAGGCTTTACACTTTA-3' and P13X:5'-CCGGATCCGTGATGNNNTTTATACAGGCG-3' as primers (NNN:AGC in case of using pG117ompPRA as a template; AGA in case of using pG117ompPRS; TTT in case of using pG117ompPRK; ACG in case of using pG117ompPRR; GTC in case of using pG117ompPRD; and TTC in case of using pG117ompPRE). A 0.3 kbp fragment (fragment 20) obtained by digesting the PCR product obtained above by PvuI and BamHI was ligated to a 0.1 kbp fragment (fragment 21) obtained by digesting pG117ompPRR by BamHI and SalI and a 3.1 kbp fragment (fragment 22) obtained by

pG117ompPRR by PvuI and SalI, and transformation was carried out. The plasmids were isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed in order to confirm that the plasmids express the target fusion proteins. These plasmids were collectively referred to as pG117ompPKX (wherein X represents one letter code of the amino acid substituted, for example, pG117ompPKA represents a plasmid with the substitution into alanine). (Fig. 12).

(2) Step 2: Preparation of fusion protein PKX

[0086] When pG117ompPKX is expressed in *E. coli*, the fusion protein PKX (Fig. 13) is expressed as inclusion body. In a case wherein OmpT protease is expressed in *E. coli*, the inclusion body is cleaved by OmpT protease merely by solubilizing with urea. To avoid this phenomenon, pG117ompPKX was transformed into the OmpT protease-deficient *E. coli* strain W3110 M25 and thus the fusion protein PKX was prepared as inclusion body.

Example 5: Cleavage of PKX by OmpT protease

[0087] It was examined whether or not the fusion protein PKX (Fig. 13) could be cleaved by OmpT protease.

[0088] PKX was reacted with the purified OmpT protease specimen at 25°C for 30 minutes. Fig. 14 shows the results of SDS-PAGE analysis thereof wherein - represents a lane free from OmpT protease; and + represents a lane of fusion protein treated with OmpT protease.

[0089] It was confirmed that PKK and PKR employed as positive controls were cleaved by OmpT protease (Fig. 14, lanes KK and KR). Also, PKA and PKS forming no basic amino acid pair were cleaved by OmpT protease (Fig. 14, lanes KA and KS). In contrast, PKD and PKE were not cleaved by OmpT protease (Fig. 14, lanes KD and KE).

[0090] To identify the cleavage sites, the peptide digestion products were isolated by HPLC after the OmpT protease treatment and the N-terminal amino acid sequences were then determined. The OmpT protease cleavage sites thus identified are listed in Table 2.

[0091] PKK, PKR, PKA and PKS, which was cleaved by OmpT protease, showed cleavage at -K↓X- (Table 2).

Table 2

OmpT protease cleavage site of fusion protein PKX	
PKX	Cleavage site
PKA	LYK↓AHHGSG (SEQ ID NO:38)
PKS	LYK↓SHHGSG (SEQ ID NO:39)
PKK	LYK↓KHHGSG (SEQ ID NO:40)
PKR	LYK↓RHHGSG (SEQ ID NO:41)
PKD	LYKDHHGSG (SEQ ID NO:42)
PKE	LYKEHHGSG (SEQ ID NO:43)
↓ stands for an OmpT protease cleavage site. The region from leucine at the 138-position (from the N-terminus) to glycine at the 146-position in the fusion protein PKX is shown as the amino acid sequence at the cleavage site.	

[0092] Accordingly, the results of Examples 3 and 5 indicate that there exist OmpT protease cleavage sites not only in the case of basic amino acid pairs (-RR-,-RK-,-KR- and -KK-) but also in the case of pairs including one basic amino acid (-RX-,-KX-). When X is aspartic acid or glutamic acid (an acidic amino acid) or proline (an imino acid), however, no cleavage arises in this site.

Example 6: Preparation of fusion proteins PRhANP and PRhCT

[0093] The results of Example 3 indicated that OmpT protease can cleave -R↓X- (wherein X represents an amino acid (17 types in total) other than aspartic acid, glutamic acid (acidic amino acids) and proline) in the amino acid sequences in the vicinity of the OmpT protease cleavage sites shown in Example 3. Also, similar results were obtained concerning the cleavage at -K↓X- in Example 5.

[0094] With respect to the recognition of substrate by this enzyme, however, it seems insufficient to merely examine

on the amino acid sequences reported hitherto and the amino acid sequences in the vicinity of the cleavage sites (i.e., in the N- and C-terminal sides) are also important. In the above Examples, the present inventors substituted the amino acid at the +1-position of the cleavage site by this enzyme. In view of the fact that the amino acid sequences in the vicinity of the cleavage sites might be important in the substrate recognition and cleavage, the present inventors further examined how the cleavage by OmpT protease occurred in the case of the target peptide moiety of the fusion proteins employed into Examples 3 and 5 was substituted with other peptides (i.e., altering the amino acid sequence in the C-terminal side from the amino acid at the +1-position).

[0095] A fusion protein PRhANP (Fig. 16), wherein α-hANP (α-type human atrial natriuretic peptide) was arranged following arginine at the 140-position from the N-terminus of the fusion protein PR (Fig. 4), and another fusion protein PRhCT (Fig. 18), wherein hCT[G] (human calcitonin precursor) was provided, were constructed and reacted with OmpT protease so as to examine whether or not α-hANP and hCT[G] could be excised.

[0096] An expression plasmid pG117ompPRhANP of the fusion protein PRhANP and an expression plasmid of pG117ompPRhCT of the fusion protein PRhCT were constructed by using pG118ompPRR (Fig. 5). The fusion proteins PRhANP and PRhCT were prepared by the following three steps.

(1) Step 1: Construction of pG117ompPRhANP(Fig. 15)

[0097] The expression plasmid pG117ompPRhANP of the fusion protein PRhANP(Fig. 16), wherein α-hANP was arranged following arginine at the 140-position of the N-terminus of the fusion protein PR (Fig. 4), was constructed. PCR was carried out by using pGHα97SII ("Daichokin o shukushu toshita seirikassei peputido seisankei ni kansuru kenkyu (Study on Physiologically Active Peptide Production System with the Use of *E. coli* as Host)", Koji Magota, Doctoral Dissertation, Kyushu University, 1991) as a template P14:5'-GCGGAGCTCCGCCTGTATCGCAGCCTGCGGAGATCCAGCTG-3' and P15:5'-CTGAGTCGACTCAGTACCGG-3' as primers. The PCR product thus obtained was isolated and digested by SacI and SalI. The 0.1 kbp fragment (fragment 23) thus obtained was ligated to a 3.4 kbp fragment (fragment 24) obtained by digesting pG117ompPRR by SacI and SalI and transformation was performed. A plasmid was isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed so that it was identified as the expression plasmid of the target fusion protein. This plasmid was referred to as pG117ompPRhANP.

(2) Step 2: Construction of pG117ompPRhCT (Fig. 17)

[0098] An expression plasmid pG117ompPRhCT of the fusion protein PRhCT (Fig. 18) wherein hCT[G] was arranged following arginine at the 140-position from the N-terminus of the fusion protein PR (Fig. 4) was constructed. PCR was carried out by using pG97S4DhCT[G]R4 (Yabuta, M., Suzuki, Y. and Ohsuye, K. Appl. Microbiol. Biotechnol.42: 703-708, 1995) as a template and P16:5'-GCGGAGCTCCGCCTGTATCGCTGTGGTAACCTGAGCACCTG-3' and P17:5'-CTGAGTCGACTTAGCCCGGG-3' as primers. The PCR product thus obtained was digested by SacI and SalI. The 0.1 kbp fragment (fragment 25) thus obtained was ligated to a 3.4 kbp fragment (fragment 26) obtained by digesting pG117ompPRR using SacI and SalI and transformation was carried out. The plasmid was isolated from each clone thus obtained, and the restriction enzyme analysis and the nucleotide sequencing at the mutated site were performed so that it was identified as the expression plasmid of the target fusion protein. This plasmid was referred to as pG117ompPRhCT.

(3) Step 3: Preparation of fusion proteins PRhANP and PRhCT

[0099] pG117ompPRhANP and pG117ompPRhCT prepared above were transformed into the OmpT protease-deficient *E. coli* strain W3110 M25 to thereby give fusion protein-producing strains. By cultivating these strains, the fusion proteins PRhANP (Fig. 16) and PRhCT (Fig. 18) were prepared as an inclusion body.

Example 7: Cleavage of PRhANP and PRhCT by OmpT protease

[0100] It was examined whether or not the fusion proteins PRhANP (Fig. 16) and PRhCT (Fig. 18) could be cleaved by OmpT protease.

[0101] PRhANP and PRhCT were reacted with the purified OmpT protease specimen at 25°C for 30 minutes at pH 7.0. The figure 19 shows the results of SDS-PAGE analysis wherein - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease. As a result, PRhANP was cleaved by OmpT protease (Fig. 19, lane α-hANP), while PRhCT was not cleaved thereby (Fig. 19, lane hCT).

[0102] Furthermore, in order to identify the cleavage site of PRhANP, the peptide digestion product after the OmpT protease treatment was isolated by HPLC and the N-terminal amino acid sequence was determined. Thus, it was con-

firmed that α-hANP had been excised.

[0103] Taking the results of this Example into consideration, it is obvious that α-hANP having serine as the N-terminal amino acid was excised from the fusion protein employed, while hCT[G] having cysteine as the N-terminal amino acid was not cleaved. In the results of Example 3, PRC having cysteine at the +1-position could be cleaved by OmpT protease but hCT[G] was not cleaved. Therefore, it has been confirmed that the cleavage by this enzyme does not depend merely on the amino acid sequence at the -1- and +1-positions of the cleavage site but is largely affected by the amino acid sequence in the vicinity of the cleavage site.

Example 8: Preparation of fusion protein RShANP

[0104] The fusion protein RShANP (Fig. 21) encoded by the expression plasmid pGRShANP (Fig. 20) is a fusion protein wherein β-gal97S originating in 97 amino acids from the N-terminus of *E. coli* β-galactosidase, serving as a protective protein, is ligated to α-hANP via a linker consisting of three amino acids (glutamine-phenylalanine-arginine). In the course of studies on OmpT protease, the present inventors found out that the fusion protein RShANP is cleaved by OmpT protease at the bond between arginine in the linker sequence and serine at the N-terminus of α-hANP. The fusion protein RShANP was prepared by the following two steps.

(1) Construction of pGRShANP (Fig. 20)

[0105] The pGHα97SII is a plasmid constructed as a β-gal97S/α-hANP fusion protein expression plasmid. A plasmid pGRShANP expressing the fusion protein RShANP, wherein lysine located immediately before the N-terminal serine of the fusion protein α-hANP expressed by pGHα97SII was converted into arginine, was constructed in the following manner.

[0106] PCR was carried out by pGHα97SII as a template and P18:5'-TACGATGCGCAATTCCGTAGCCTGCGG-3' and P19:5'-TGCCTGACTGCGTTAGCAATTTAACTGTGAT-3' as primers and thus 0.2 kbp PCR product (P20) wherein lysine located as the amino acid codon immediately before the N-terminal serine of α-hANP had been converted into arginine was obtained.

[0107] Then, PCR was carried out again by using the thus obtained PCR product (P20) and P21:5'-TTATCGCCACTGGCAGCAGC-3' as primers and pGHα97SII as a template to thereby give a 1.0 kbp PCR product containing a linker DNA sequence with the substitution by arginine. This 1.0 kbp PCR product was digested by BglII and EcoRI and thus a 0.2 kbp DNA fragment (Fragment 27) was isolated. The α-hANP expression plasmid pGHα97SII was digested by BglII and EcoRI and the 3.0kbp fragment (fragment 28) thus obtained was ligated to the fragment 27, thereby constructing pGRShANP.

(2) Step 2: Preparation of fusion protein RShANP

[0108] When pGRShANP is expressed in *E. coli*, the fusion protein RShANP (Fig. 21) is expressed as inclusion body. In a case wherein OmpT protease is expressed in *E. coli*, the inclusion body is cleaved by OmpT protease merely by solubilizing with urea. To avoid this phenomenon, pGRShANP was transformed into the OmpT protease-deficient *E. coli* strain W3110M25 and thus the fusion protein RShANP was prepared as an inclusion body.

Example 9: Cleavage of RShANP by OmpT protease

[0109] The physiologically active peptide α-hANP was excised from the fusion protein RShANP (Fig. 21) in the following manner. RShANP was reacted with OmpT protease at pH7.0 at 37°C for 2 hours followed by SDS-PAGE analysis. The result is shown in Fig. 24 (lane RS) wherein - represents a lane free from OmpT protease; and + represents a lane with the addition of OmpT protease. Based on this result, it was confirmed that RShANP could be cleaved by OmpT protease. To identify the cleavage site, the peptide digestion product obtained after the OmpT protease reaction was isolated by HPLC and the N-terminal amino acid sequence was determined. The OmpT protease cleavage site thus identified is listed in Table 3 (RShANP). As Table 3 shows, RShhANP was cleaved by OmpT protease at -AQFR↓SLRR- and thus the physiologically active peptide α-hANP was directly excised. Also, cleavage at -AQFRSLR↓R-was partly detected and the excision of α-hANP(3-28) was also confirmed.

Table 3

OmpT protease cleavage sites of fusion proteins RShANP and RXhANP	
RXhANP	Cleavage site
RShANP	QFR↓SLRRS (SEQ ID NO:52)
RRhANP	QFR↓RLRRS (SEQ ID NO:53)
RAhANP	QFR↓ALRRS (SEQ ID NO:54)
RChANP	QFR↓CLRRS (SEQ ID NO:55)
↓ stands for an OmpT protease cleavage site. The region from glutamine at the 99-position (from the N-terminus) to serine at the 106-position in the fusion protein RShANP or RXhANP is shown as the amino acid sequence at the cleavage site.	

Example 10: Preparation of fusion protein RXhANP

[0110] In case of using the fusion protein PRX as a substrate, it was confirmed that the amino acid sequence -RLYRXHHG-(wherein X represents an amino acid selected from the 20 types) having as X an amino acid other than aspartic acid and glutamic acids (i.e., acidic amino acids) and proline (i.e., an imino acid) was cleavable by OmpT protease. Thus, it was examined whether or not cleavage similar to PRX arose in the OmpT protease cleavage site of other amino acid sequences. First, an expression plasmid pGRXhANP (wherein X is R, A or C) of the fusion protein RXhANP (wherein X is R, A or C), which had been constructed by transferring mutation into the expression plasmid pGRShANP (Fig. 20) to thereby convert the OmpT protease cleavage site -AQFR↓SLRR- into -AQFRXLRR- (wherein X is arginine, alanine or cysteine), was constructed (Fig. 22). With respect to X, arginine was selected as a positive control forming a basic amino acid pair. Alanine and cysteine were selected because they provided relatively high cleavage efficiency in Example 3. RXhANP (Fig. 23) was constructed by the following two steps.

(1) Step 1: Construction of pGRXhANP (Fig. 22)

[0111] pGRXhANP was constructed by introducing a mutation into pGRShANP. pGRShANP was employed as a template, while P3:5'-ACCCCAGGCTTTACACTTTA-3' and P22X:5'-TCTCCGCAGNNNACGGAATTGCGCATCGTA-3' (NNN:AGC in case of converting into alanine; GCA in case of converting the resine into cysteine; and ACG in case of converting into arginine) were employed as primers. From the PCR product thus obtained, the target PCR product was isolated (PCR product 29). Similarly, PCR was carried out by using pGRShANP as a template and P23X:5'-CAATTC-CGTNNNCTGCGGAGATCCAGCTGC-3' (NNN:GCT in the case of converting into alanine; TGC in the case of converting into cysteine; and CGT in the case of converting into arginine) and P24:5'-GCCTGACTGCGTTAGCAATTTAACTGTGAT-3' as primers and the target PCR product (PCR product 30) was isolated. PCR was carried out by using the PCR products 29 and 30 obtained above as templates and P3:5'-ACCCCAGGCTT-TACACTTTA-3' and P24:5'-GCCTGACTGCGTTAGCAATTTAACTGTGAT-3' as primers. The PCR product was collected and digested by EcoRI and BglII to thereby isolate a 0.2 kbp DNA fragment (fragment 31). This fragment 31 was ligated to a 30 kbp fragment (Fragment 32), which had been obtained by digesting pGRShANP with EcoRI and BglII, and transformation was performed. A plasmid was isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed so that it was identified as the expression plasmid of the target fusion protein. This plasmid was referred to as pGRXhANP (wherein X represents one letter code of the amino acid converted; namely, a fusion protein having substitution into alanine is expressed as pGRAhANP).

(2) Step 2: Preparation of fusion protein RXhANP

[0112] When pGRXhANP is expressed in *E. coli*, the fusion protein RXhANP (Fig. 23) is expressed as inclusion body. In a case where OmpT protease is expressed in *E. coli*, the inclusion body is cleaved by OmpT protease merely by dissolving with urea. To avoid this phenomenon, therefore, pGRXhANP was transformed into the OmpT protease-deficient *E. coli* strain W3110 M25 and thus the fusion protein RXhANP was prepared in the form of inclusion body.

Example 11: Cleavage of RXhANP by OmpT protease

[0113] The fusion protein RXhANP (Fig. 23), wherein the OmpT protease -AQFR↓SLRR- of the fusion protein RShANP (Fig. 21) had been converted into -AQFRXLRR-(wherein X is arginine, alanine or cysteine) was treated with OmpT protease at pH7.0, 37°C for 2 hours. Fig. 24 shows the result of SDS-PAGE analysis.

[0114] Similarly to the cases of using PRR, PRA and PRC as a substrate, cleavage by OmpT protease was observed in RRhANP, RAhANP and RChANP. To identify the cleavage sites, the peptide digestion products were isolated by HPLC after the OmpT protease treatment and the N-terminal amino acid sequences were determined. The OmpT protease cleavage sites thus identified are listed in Table 3. As Table 3 shows, RRhANP, RAhANP and RChANP were each cleaved by OmpT protease at -AQFRK↓XLRR-. Furthermore, taking the results in the fusion protein PRX into consideration too, it is suggested that there exist OmpT protease cleavage sites comprising one basic amino acid in the case wherein the amino acid sequence in the vicinity the OmpT protease cleavage site is altered.

[0115] In this Example, arginine-arginine alone was slightly cleaved, among the four sites (arginine-arginine, arginine-methionine, arginine-isoleucine and arginine-tyrosine) existing in α-hANP molecule consisting of 28 amino acids, while the other bonds were scarcely cleaved. These facts suggest that the cleavage sequences reported hitherto and the sequences proposed by the present inventors (i.e., arginine-X or lysine-X wherein X is one of the 17 amino acids other than aspartic acid and glutamic acid (acidic amino acids) and proline (an imino acid)) alone are not sufficient to be cleaved by OmpT protease. That is to say, it is indicated that a method for creating a novel cleavage site by utilizing a region containing the known cleavage sites of this enzyme, as performed by the present inventors, is industrially useful.

Example 12: Preparation of fusion protein PRRXA

[0116] As described above, it is pointed out that OmpT protease cleaves the central bond of arginine-X or lysine-X wherein X is one of the 17 amino acids other than aspartic acid and glutamic acid (acidic amino acids) and proline (an imino acid). However, OmpT protease does not always cleave all of these bonds in proteins and peptides. It is estimated that the cleavage by OmpT protease is largely affected by the amino acid sequences in the vicinity of the cleavage sites. It is rather considered that this enzyme has high substrate specificity because it cleaves exclusively specific sites. The present inventors expected that the substrate specificity of this enzyme might be further clarified by examining the effect of the amino acid sequence in the N-terminal side of the known cleavage site of the enzyme and, therefore, conducted the following experiment.

[0117] The fusion protein PRR (shown in Fig. 25) having a structure cleavable by OmpT protease consists of a protective protein (β-gal117S4H) originating in 117 amino acids from the N-terminus of *E. coli* β-galactosidase and human glucagon-like peptide-1 (GLP-1[G]). As shown in Fig. 25, a fusion protein PRRXA (wherein X corresponds to the position of the amino acid of the cleavage site and represented in -1, -2 ----- -10 excluding -7), wherein an amino acid in the amino acid sequence at the -10- to -1-positions (i.e., GYDAELRLYR) of the OmpT protease cleavage site -QMHGY-DAELRLYR↓RHHG- existing in the linker peptide of the fusion protein PRR had been converted one by one into alanine, was prepared and the cleavage of these fusion proteins by OmpT protease was examined.

[0118] The fusion protein PRRXA was prepared by the following two steps.

(Step 1) Construction of pG117ompPRRXA (Figs. 26, 27, 28, 29)

[0119] The expression plasmid of the fusion protein PRRXA was referred to as pG117ompPRRXA (corresponding to the fusion protein PRRXA). However, alanine at the -7-position was not substituted. These substitutions were carried out by introducing DNA mutations into pG117ompPRR.

[0120] The PCR method was employed in order to induce mutation. As shown in Fig. 26, the plasmids pG117ompPRR-2A, pG117ompPRR-3A and pG117ompPRR-4A were constructed by using pG117ompPRR as a template and P10: 5'-TGCCGAGGATGACGATGAGC-3', P25: 5'-GCGGAGCTCCGCCTGGCTCGCCGTCATCAC-3', P26: 5'-GCGGAGCTCCGCGCTTATCGCCGTCATCAC-3' and P27: 5'-GCGGAGCTCGCTCTGTATCGCCGTCATCAC-3' as primers. The PCR products obtained by using the combinations of the primers P10/P25, P10/P26 and P10/P27 were digested by SacI and KpnI to give each 0.1 kbp fragment (fragment 33) in each combination. Separately, pG117omoPRR was digested by BglII and SacI to give a 0.2 kbp fragment (fragment 34). These fragments 33 and 34 were ligated to a 3.2 kbp fragment (fragment 35) obtained by digesting pG117ompPRR by BglII and KpnI and transformation was carried out. A plasmid was isolated from each clone thus obtained.

[0121] As shown in Fig. 27, expression plasmids pG117ompPRR-5A and pG117ompPRR-6A were constructed by using the pG117ompPRR as a template and P10, P28: 5'-CAGATGCATGGTTATGACGCGGAGGCTCGC-3', and P29: 5'-CAGATGCATGGTTATGACGCGGCTCTCCGC-3' as primers. The PCR product obtained by using the combinations of the primers P10/P28 and P10/P29 was digested by NsiI and KpnI to give a 0.1 kbp fragment (fragment 36) in each

combination. Separately, pG117ompPRR was digested by NsiI and KpnI to give a 3.4 kbp fragment (fragment 37). These fragments 36 and 37 were ligated together and transformation was carried out. A plasmid was isolated from each clone thus obtained.

[0122] Further, expression plasmids pG117ompPRR-8A, pG117ompPRR-9 and pG117ompPRR-10A were constructed as shown in Fig. 28. pG117ompPRR was used as a template and P3: 5'-ACCCCAGGCTTTACACTTTA-3' P30: 5'-GCGGAGCTCCGCAGCATAACCATGCATCTG-3', P31: 5'-GCGGAGCTCCGCGTCAGCACCATGCATCTG-3' and P32: 5'-GCGGAGCTCCGCGTCATAAGCATGCATCTG-3' were used as primers. The PCR products obtained by the combinations of the primers P3/P30, P3/P31 and P3/P32 were digested by SacI and BglII to give a 0.2 kbp fragment (fragment 38) in each combination. Separately, pG117ompPRR was digested by KpnI and SacI to give a 0.1 kbp fragment (fragment 39). These fragments 38 and 39 were ligated to a 3.2 kbp fragment (fragment 40) obtained by digesting pG117ompPRR with BglII and KpnI and transformation was carried out. A plasmid was isolated from each clone thus obtained.

[0123] pG117ompPRR-1A was constructed as shown in Fig. 29 by using pG117ompPRR as a template and P10 and P33: 5'-GCGGAGCTCCGCCTGTATGCTCGTCATCAC-3' as primers. The PCR product obtained by the combination of the primers P10/P33 was digested by SacI to give a 0.1 kbp fragment (fragment 41). Separately, pG117ompPRR was digested by SacI to give a 3.4 kbp fragment (fragment 42). These fragments 41 and 42 were ligated together and transformation was carried out. A plasmid was isolated from each clone thus obtained.

[0124] These expression plasmids pG117ompPRRXAs thus constructed were all subjected to restriction enzyme analysis and nucleotide sequencing at the mutation site and thus confirmed as being the expression plasmids of the target fusion proteins PRRXAs.

(Step 2) Preparation of fusion proteins PRR and PRRXA

[0125] When pG117ompPRR and pG117ompPRRXA are expressed in *E. coli*, the fusion proteins PRR and PRRXA are expressed as inclusion bodies. In a case where OmpT protease is expressed in *E. coli*, the inclusion body is cleaved by OmpT protease merely by dissolving with urea. To avoid the cleavage, therefore, pG117ompPRR and pG117ompPRRXA were transformed into the OmpT protease-deficient *E. coli* strain W3110M25 and thus the fusion proteins PRR and PRRXA were prepared in the form of inclusion body.

Example 13: Cleavage of fusion proteins PRR and PRRXA by OmpT protease

[0126] PRR and PRRXA were reacted with the purified OmpT protease specimen at 25°C for 60 minutes at pH 7.0. After the completion of the enzymatic reaction, SDS-PAGE analysis was carried out. The results are shown in Fig. 30.

[0127] The PRR-1A was not cleaved by OmpT protease (Fig. 30, lane 1A). Although the cleavage by OmpT protease was confirmed in the fusion proteins other than PRR-1A, the amount of the 4.9 kDa peptide fragment formed by the cleavage differed from protein to protein.

[0128] In order to quantitate the 4.9 kDa peptide fragment, the liquid reaction mixture of the above-described OmpT protease reaction was subjected to HPLC. In the OmpT protease reaction of each of the fusion proteins other than PRR-1A, a peak was detected at a retention time of 8.8 minutes. The N-terminal amino acid sequence of this peak at 8.8 minutes detected in PRR had been determined and it had been clarified as a 4.9 kDa peptide fragment formed by the cleavage at -QMHGYDAELRLYR↓RHHG- (Table 4). Thus, it is assumed that the peak at 8.8 minutes formed by reacting each fusion protein with OmpT protease corresponds to the 4.9 kDa peptide fragment.

[0129] The relative peak area at the retention time of 8.8 minutes indicates the amount of the 4.9 kDa peptide fragment formed by the cleavage. Table 4 shows the data of the relative amounts of the 4.9 kDa peptide fragment calculated by referring the amount in the case of PRR as to 100. Excluding PRR-1A, PRR-4A showed the smallest amount of the 4.9 kDa peptide fragment and PRR-6A showed the largest one. Based on these results, it is considered that the -4- and -6-positions (other than -1-position) largely affect the cleavage by OmpT protease.

Table 4

Cleavage of fusion protein PRRXA (X ranging from -10- to -1, excluding -7)	
PRRXA	Relative amount of 4.9 kDa peptide
PRR	100
PRR-1A	ND

Table 4 (continued)

Cleavage of fusion protein PRRXA (X ranging from -10- to -1, excluding -7)	
PRRXA	Relative amount of 4.9 kDa peptide
PRR-2A	150
PRR-3A	44
PRR-4A	24
PRR-5A	89
PRR-6A	330
PRR-8A	200
PRR-9A	160
PRR-10A	160

[0130] The 4.9 kDa peptide is formed by the cleavage of the fusion proteins by OmpT protease. The amount of the 4.9 kDa peptide of PRR is referred to as 100. ND means not detectable.

Example 14: Preparation of fusion proteins PRR-4X and PRR-6X

[0131] The OmpT protease is an endoprotease which recognizes and cleaves mainly basic amino acid pairs. The substrate specificity of mammalian furin, which is also an endoprotease recognizing and cleaving basic amino acid pairs (cleaving the C-terminal side of basic amino acid pairs), has been studied in detail and it is reported that furin recognizes arginine at the -1-position and basic amino acids at the -2-, -4- and -6-positions concerning the cleavage site.

[0132] The results of Example 13 indicate that the substitution of arginine which is the basic acid at the -4-position into alanine makes the cleavage by OmpT protease difficult, while the substitution of glutamic acid which is the acidic amino acid at the -6-position into alanine facilitates the cleavage by OmpT protease.

[0133] Based on these facts, it was assumed that the cleavage by the OmpT protease might be affected by the charges on the amino acids at the -6- and -4-positions of the cleavage site. Thus, the cleavage by OmpT protease was examined by forming fusion proteins wherein the amino acids at these positions were substituted by arginine and lysine (basic amino acids), aspartic acid and glutamic acid (acidic amino acids) and asparagine and glutamine (neutral amino acids being similar in structure to acidic amino acids).

[0134] A fusion protein with the substitution at the -4-position was referred to PRR-4X (Fig. 31), while a fusion protein with the substitution at the -6-position was referred to PRR-6X (Fig. 32), wherein X represents one letter of the amino acid at the -4- or -6-position. The fusion proteins PRR-4X and PRR-6X were prepared by the following two steps.

(Step 1) Construction of pG117ompPRR-4X and pG117ompPRR-6X (Figs. 33 and 34)

[0135] The expression plasmid of the fusion protein PRR-4X, wherein arginine at the -4-position of the OmpT protease cleavage site -QMHGYDAELRLYR↓RHHG- of the fusion protein PRR had been substituted by lysine (a basic amino acids), aspartic acid or glutamic acid (an acidic amino acid) or asparagine or glutamine (a neutral amino acid being similar in structure to acidic amino acids), was referred to as pG117ompPRR-4X, while the expression plasmid of the fusion protein PRR-6X, wherein glutamic acid at the -6-position had been substituted in the same manner, as to pG117ompPRR-6X. These substitutions were carried out by introducing DNA mutations into pG117ompPRR.

[0136] The mutations were introduced by PCR. Expression plasmids pG117ompPRR-4K, pG117ompPRR-4D, pG117ompPRR-4E, pG117ompPRR-4N and pG117ompPRR-4Qwere were constructed by the procedure shown in Fig. 33. pG117ompPRR was used as a template, while P10, P34: 5'-GCGGAGCTCAAACTGTATCGCCGTCATCAC-3', P35: 5'- GCGGAGCTCGACCTGTATCGCCGTCATCAC-3' P36: 5'- GCGGAGCTCGAACTGTATCGCCGTCATCAC -3', P37: 5'- GCGGAGCTCAACCTGTATCGCCGTCATCAC -3' and P38: 5'- GCGGAGCTCCAGCTGTATCGCCGTCATCAC -3' were used as primers. As the PCR products with the use of the combinations of the primers P10/P34, P10/P35, P10/P36, P10/P37 and P10/P38, the 0.3 kbp fragment (fragment 43) was obtained. PCR was carried out again by using pG117ompPRR as a template and P3 and the fragment 43 as primers to give the 0.8 kbp fragment (fragment 44). The 0.1 kbp fragment (fragment 45) obtained by digesting fragment 44 with NsiI and KpnI was ligated to the 3.4 kbp fragment (fragment 46) obtained by digesting pG117ompPRR with NsiI and KpnI, and transformation was carried out. A

plasmid was isolated from each clone thus obtained.

As shown in Fig. 34, expression plasmids pG117ompPRR-6R, pG117ompPRR-6K, pG117ompPRR-6D, pG117ompPRR-6N and pG117ompPRR-6Q were constructed by using pG117ompPRR as a template and P10, P39: 5'-CAGATGCATGGTTATGACGCGCGTCTCCGC-3', P40: 5'- CAGATGCATGGTTATGACGCGAAACTCCGC-3', P41: 5'- CAGATGCATGGTTATGACGCGGACCTCCGC-3', P42: 5'- CAGATGCATGGTTATGACGCGAACCTCCGC-3' and P43: 5'- CAGATGCATGGTTATGACGCGCAGCTCCGC-3' as primers. The PCR products obtained by using the combinations of the primers P10/P39, P10/P40. P10/P41, P10/P42 and P10/P43 were digested by NsiI and KpnI to give a 0.1 kbp fragment (fragment 46). Further, pG117ompPRR was digested by NsiI and KpnI to give the 3.4 kbp fragment (fragment 47). These fragments 46 and 47 were ligated together and transformation was carried out. A plasmid was isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed. Thus, these plasmids were identified as the expression plasmids pG117ompPRR-4X and pG117ompPRR-6X of the target fusion proteins PRR-4X and PRR-6XX.

(Step 2) Preparation of fusion proteins PRR-4X and PRR-6X

[0137] When pG117ompPRR-4X and pG117ompPRR-6X are expressed in *E. coli,* the fusion proteins PRR-4X and PRR-6X (Figs. 31 and 32) are expressed as inclusion body. In a case where OmpT protease is expressed in *E. coli,* these inclusion body is cleaved by OmpT protease merely by dissolving with urea. To avoid the cleavage, therefore, pG117ompPRR-4X and pG117ompPRR-6X were transformed into the OmpT protease-deficient *E. coli* strain W3110M25 and thus the fusion proteins PRR-4X and PRR-6X were prepared in the form of inclusion body.

Example 15: Cleavage of fusion proteins PRR-4X and PRR-6X by OmpT protease

[0138] PRR-4X was reacted with the purified OmpT protease specimen at 25°C for 60 minutes at pH 7.0. After the enzymatic reaction, SDS-PAGE analysis was performed. Fig. 35A shows the results wherein - represents a lane free from OmpT protease, and + represents a lane with the addition of OmpT protease (2.0 U/ml).

[0139] Although the cleavage by OmpT protease was observed in all of the fusion proteins, the amount of the 4.9 kDa peptide fragment formed by the cleavage differed from protein to protein.

[0140] Then the 4.9 kDa peptide fragment was quantitated by using HPLC. In the cases of adding OmpT protease, peaks were detected at retention time of 8.8 minutes. As described in Example 13, these peaks are seemingly assignable to the 4.9 kDa peptide fragment.

[0141] Table 5 shows the data of the relative amounts of the 4.9 kDa peptide fragment calculated by referring the amount in the case of PRR as to 100. The relative amounts of the peptide fragment formed by the cleavage were from 2 to 3% (PRR-4D and PRR-4E) or from 20 to 50% (PRR-4A, PRR-4N and PRR-4Q) or almost comparable to PRR (PRR-4K). Based on these results, it is considered that OmpT protease recognizes the electrical charge of the amino acid at the -4-position

Table 5

Cleavage of fusion protein PRR-4X (X being K, A, N, Q, D or E)	
PRR-4X	Relative amount of 4.9 kDa peptide
PRR	100
PRR-4K	96
PRR-4A	49
PRR-4N	48
PRR-4Q	23
PRR-4D	2.8
PRR-4E	2.0

[0142] The 4.9 kDa peptide is formed by the cleavage of the fusion proteins by OmpT protease. The amount of the 4.9 kDa peptide of PRR is referred to as 100. PRR has Arg at the -4-position.

[0143] As shown in Fig. 32, the cleavage by OmpT protease was further examined by using the fusion protein PRR-6X wherein glutamic acid at the -6-position of the fusion protein PRR had been substituted. PRR-6X was reacted with the purified OmpT protease specimen at 25°C for 60 minutes at pH 7.0. After the enzymatic reaction, SDS-PAGE analysis was performed. Fig. 35B shows the results wherein - represents a lane free from OmpT protease, and + represents a lane with the addition of OmpT protease (0.1 U/ml).

[0144] Although the cleavage by OmpT protease was observed in all of the fusion proteins, the amount of the 4.9 kDa peptide fragment formed by the cleavage differed from protein to protein similar to the case of PRR-4X.

[0145] Then the 4.9 kDa peptide fragment was quantitated by using HPLC. Table 6 shows the data of the relative amounts of the 4.9 kDa peptide fragment calculated by referring the amount in the case of PRR as to 100. The relative amounts of the peptide fragment formed by the cleavage were almost comparable to PRR (PRR-6D), from about 3 to 4 times as much as PRR (PRR-6A, PRR-6N and PRR-6Q) or about 10 times as much as PRR (PRR-6R and PRR-6K). Based on these results, it is considered that OmpT protease recognizes also the electrical charge of the amino acid at the -6-position too.

Table 6

Cleavage of fusion protein PRR-6X (X being R, K, A, N, Q or D)	
PRR-6X	Relative amount of 4.9 kDa peptide
PRR	100
PRR-6R	1000
PRR-6K	1400
PRR-6A	310
PRR-6N	430
PRR-6Q	390
PRR-6D	110

[0146] The 4.9 kDa peptide is formed by the cleavage of the fusion proteins by OmpT protease. The amount of the 4.9 kDa peptide of PRR is referred to as 100. PRR has Glu at the -6-position.

[0147] These results suggest that OmpT protease recognizes the amino acids at the -4- and -6-positions in the digested site of the substrate and the cleavage ratio is elevated in the case wherein the amino acids at these positions are basic amino acids but lowered in the case wherein the amino acids at these positions are acidic amino acids.

Example 16: Application to sequence cleavable by OmpT protease

[0148] Based on the results of Example 15, it is assumed that the cleavage efficiency of OmpT protease can be elevated by converting the amino acids at the -4- and -6-positions of the known OmpT protease cleavage site into basic amino acids. From this viewpoint, a fusion protein RShANPR (Fig. 36) was formed by substituting the amino acids at the -4- and -6-positions of the OmpT protease cleavage site of the fusion protein RShANP (Fig. 21), having a structure cleavable by OmpT protease and thus releasing α-hANP, by arginine (a basic amino acid) and then it was examined by the following three steps whether or not these fusion proteins differed from each other in the cleavage by OmpT protease.

(Step 1) Construction of pGRShANPR (Fig. 37)

[0149] The expression plasmid of the fusion protein RShANPR, wherein alanine at the -4-position and tyrosine at the -6-position of the OmpT protease cleavage site -QMHGYDAQFR↓SLRR- of the fusion protein RShANP had been substituted each by arginine, was referred to as pGRShANPR. These conversions were carried out by introducing DNA mutations into pGRShANP.

[0150] The DNA mutations were introduced by PCR. As shown in Fig. 37, pGRShANP was used as a template and P10 and P44: 5'-ATGCACGGTCGTGATCGTCAATTCCGTAGC-3' were used as primers. As the product of PCR with the use of the combinations of the primers P10/P44, a 0.3 kbp fragment (fragment 48) was obtained. Then PCR was

carried out again by using pGRShANP as a template and the P3 and the fragment 48 as primers to give the 0.6 kbp fragment (fragment 49). The 0.2 kbp fragment (fragment 50) obtained by digesting fragment 49 with BglII and EcoRI was ligated to the 3.0 kbp fragment (fragment 51) obtained by digesting pGRShANP with BglII and EcoRI, and transformation was carried out. A plasmid was isolated from each clone thus obtained and restriction enzyme analysis and nucleotide sequencing at the mutated site were performed so that it was identified as the expression plasmid pGRShANPR of the target fusion protein RShANPR.

(Step 2) Preparation of fusion proteins RShANP and RShANPR

[0151] When pGRShANP and pGRShANPR are expressed in *E. coli*, the fusion proteins RShANP and RShANPR (Fig. 36) are expressed as inclusion body. In a case where OmpT protease is expressed in *E. coli*, these inclusion body is cleaved by OmpT protease merely by dissolving with urea. To avoid the cleavage, therefore, pGRShANP and pGRShANPR were transformed into the OmpT protease-deficient *E. coli* strain W3110M25 and thus the fusion proteins RShANP and RShANPR were prepared in the form of inclusion body.

(Step 3) Cleavage of fusion proteins RShANP and RShANPR by OmpT protease

[0152] RShANP and RShANPR were reacted with the purified OmpT protease specimen at 25°C for 90 minutes at pH 7.0. After the completion of the enzymatic reaction, the peptide fragment thus released was quantitated by HPLC. In the case of adding OmpT protease (2.0 U/ml), a peak was detected at the retention time of 4.7 minutes. By isolating this peak and determining the N-terminal amino acid sequence, it was identified as α-hANP.

[0153] The relative peak area at the retention time of 4.7 minutes (i.e., the relative amount of the released α-hANP) of RShANPR was 2.2 times as much as that of RShANP. Based on these results, it is expected that the cleavage efficiently by OmpT protease can be elevated by converting the amino acids at the -6- and -4-positions of the known OmpT protease cleavage site into basic amino acids (arginine in the above case).

EFFECTS OF THE INVENTION

[0154] In one aspect of the method according to the present invention, use is made of the properties of OmpT protease that it shows a highly specific effect of cleaving exclusively the bonds between arginine-X and lysine-X (wherein X represents an amino acid other than glutamic acid, aspartic acid or proline) located in specific amino acid sequences. Therefore, use of OmpT protease makes it possible to, for example, select a peptide constructing from amino acids other than glutamic acid, aspartic acid or proline as the N-terminal amino acid in the case of excising the target peptide from a fusion protein expressed by genetic engineering techniques, and to avoid the cleavage at undesired peptide bonds by converting the amino acid at the +1-position into glutamic acid, aspartic acid or proline.

[0155] In another aspect of the method according to the present invention, use is made of another properties of the OmpT protease that it recognizes the charges of the amino acids at the -6- and -4-positions. In case of excising the target peptide from a fusion protein expressed by genetic engineering techniques similar to the above-described case, the cleavage ratio can be elevated by converting the amino acids at the -6- and -4-positions into basic amino acids and the cleavage at undesired peptide bonds can be minimized by converting the amino acids at the -6- and -4-positions into acidic amino acids. When a fusion protein is expressed in inclusion body, OmpT protease is recovered together with the inclusion body. Therefore, the present invention is particularly effective in the case of using *E. coli* as a host.

Claims

1. A method of controlling cleavage of a polypeptide by OmpT protease which comprises converting a sequence site consisting of two arbitrary consecutive amino acids and/or amino acid(s) in the vicinity of, said site in said polypeptide into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position concerning said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that a desired part of said polypeptide is cleaved by OmpT protease and/or an undesired part of said polypeptide is not (or hardly) cleaved by ompT protease.

2. The method as claimed in claim 1 characterized by, in a case where the amino acid at the -1-position of the sequence site consisting of two arbitrary consecutive amino acids in said polypeptide is neither lysine nor arginine, converting said amino acid into lysine or arginine and setting an amino acid X (wherein X is an amino acid other than glutamic acid, aspartic acid, proline, arginine, lysine, alanine, methionine or valine) as the amino acid at the +1-position so that a desired part of the polypeptide is cleaved by OmpT protease.

3. The method as claimed in claim 1 which comprises, in a polypeptide having a sequence site consisting of two arbitrary consecutive amino acids cleavable by OmpT protease, converting the amino acids at said site and/or in the vicinity thereof into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position of said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site; so that a desired part of said polypeptide is cleavable by OmpT protease.

4. The method as claimed in claim 3 which comprises, in a polypeptide having a sequence site consisting of two arbitrary consecutive amino acids cleavable by OmpT protease, converting the amino acids at said site and/or in the vicinity thereof into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position of said site and setting an amino acid X (wherein X is an amino acid other than glutamic acid, aspartic acid or proline) as the amino acid at the +1-position; and/or (2) setting an amino acid X (wherein X is an amino acid other than acidic amino acids) as the amino acid(s) at the -4-position and/or the -6-position in said sequence.

5. A method as claimed in claim 1, 3 or 4 characterized in that the amino acid(s) at the -4-position and/or the -6-position are basic amino acid(s).

6. The method as claimed in claim 5 characterized in that the amino acid(s) at the -4-position and/or the -6-position are lysine or arginine.

7. The method as claimed in claim 1 which comprises, in a polypeptide having a sequence site consisting of two arbitrary consecutive amino acids cleavable by OmpT protease, converting the amino acids at said site and/or in the vicinity thereof into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position of said site and setting a specific amino acid as the amino acid at the +1-position; and/or (2) setting specific amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position relative to said site: so that undesired parts of said polypeptide are not (or hardly) cleaved by OmpT protease.

8. The method as claimed in claim 7 which comprises, in a polypeptide having a sequence site consisting of two arbitrary consecutive amino acids cleavable by OmpT protease, converting the amino acids at said site and/or in the vicinity thereof into other amino acids, characterized by (1) setting lysine or arginine as the amino acid at the -1-position of said site and setting an amino acid X (wherein X is glutamic acid, aspartic acid or proline) as the amino acid at the +1-position; and/or (2) setting acidic amino acid(s) as the amino acid(s) at the -4-position and/or the -6-position in said sequence.

9. A method as claimed in any of claims 1 to 8 comprising expressing in host cells a gene, which encodes a fusion protein consisting of a target polypeptide fused with a protective peptide via a cleavage site (optionally located in a linker peptide) and being cleavable by OmpT protease at said cleavage site, and cleaving off the protein at said cleavage site by OmpT protease to thereby obtain the target polypeptide from said fusion protein.

10. The method as claimed in claim 9 wherein an amino acid sequence cleavable by OmpT protease exists in the amino acid sequences of the protective peptide, the linker peptide and/or the target polypeptide constituting said fusion protein.

11. A method of producing a target polypeptide which comprises expressing in host cells a gene, which encodes a fusion protein consisting of a target polypeptide fused with a protective peptide via a cleavage site (optionally located in a linker peptide) and being cleavable by OmpT protease at said cleavage site, and cleaving the protein at said cleavage site by OmpT protease to thereby obtain the target polypeptide from said fusion protein, characterized by converting the amino acids at said cleavage site and/or amino acid(s) in the vicinity of said site as described in any of claims 1 to 8.

12. The method as claimed in claim 11 wherein an amino acid sequence cleavable by OmpT protease exists in the amino acid sequences of the protective peptide, the linker peptide and/or the target polypeptide constituting said fusion protein.

13. A method as claimed in any of claims 1, 7 and 8 wherein a gene encoding a polypeptide is expressed in host cells so that said polypeptide is cleavable at an undesired part by OmpT protease.

14. A method of producing a polypeptide which comprises expressing a gene encoding the polypeptide in host cells,

characterized in that when said polypeptide is cleaved by OmpT protease at undesired parts, amino acid(s) are converted as described in any of claims 1 to 8.

15. A method as claimed in any of claims 9 to 14 wherein the host cell is Escherichia coli.

16. A method as claimed in any of claims 9 to 15 wherein said target polypeptide is a natriuretic peptide.

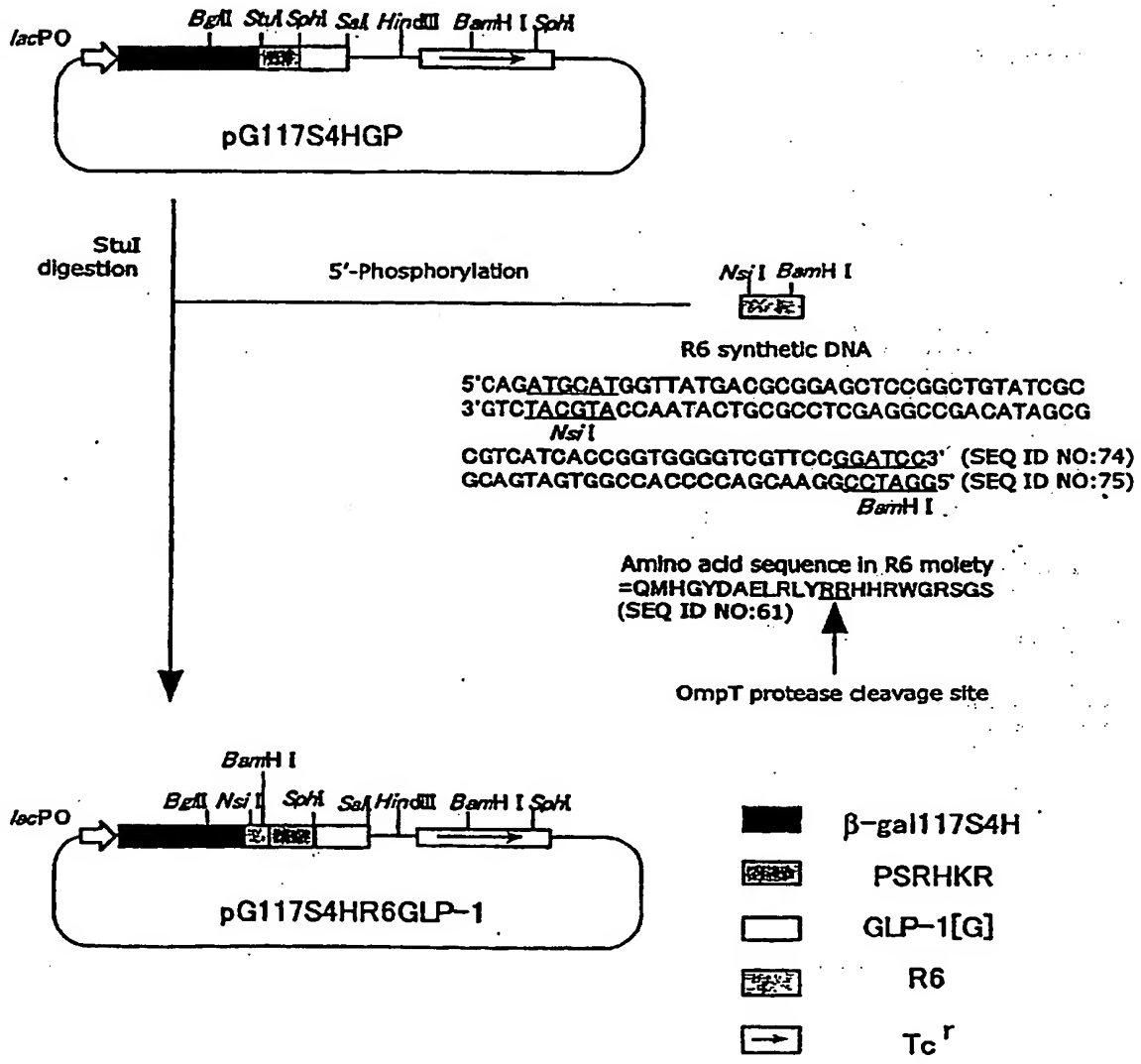


Fig. 1

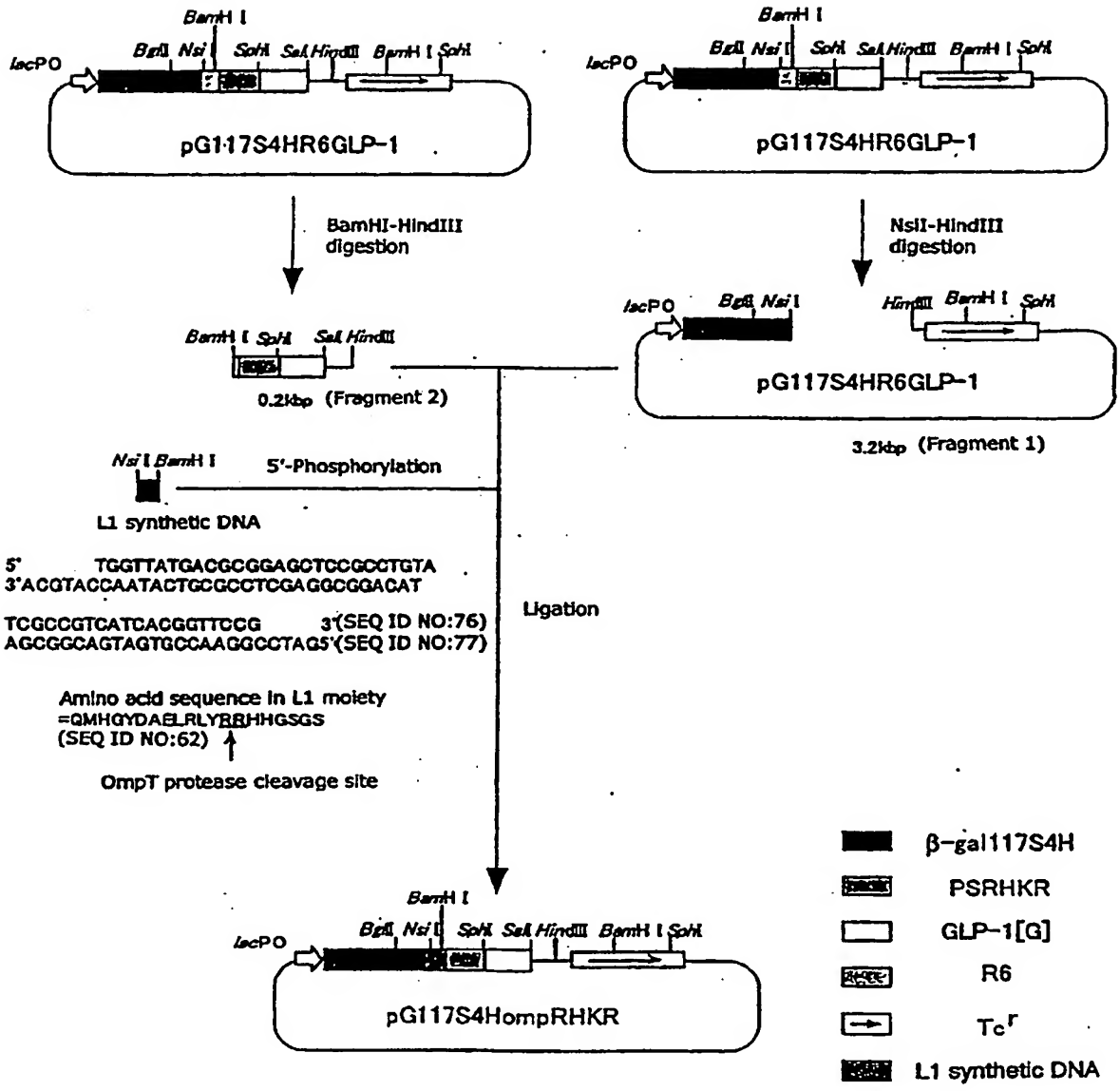


Fig. 2

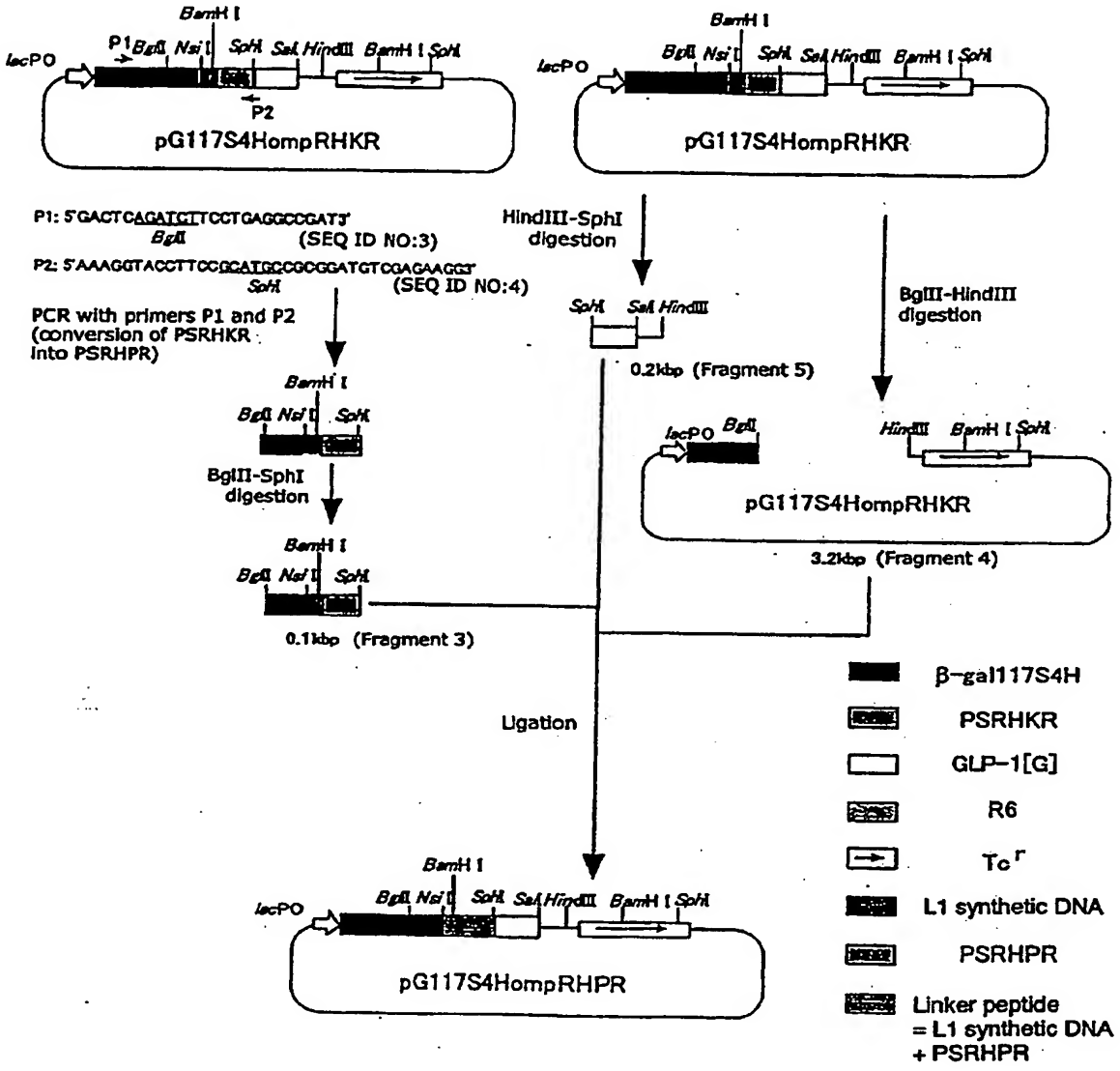


Fig. 3

15

Met Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Lys

30

Asp Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala

45

His Pro Pro Phe Ala Ser Trp Arg Asn Ser Asp Asp Ala Arg Thr

60

Asp Arg Pro Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg

75

Phe Ala Trp Phe Pro Ala Pro Glu Ala Val Pro Glu Ser Leu Leu

90

Asp Leu Pro Glu Ala Asp Thr Val Val Val Pro Asp Ser Ser Asn

105

Trp Gln Met His Gly Tyr Asp Ala Pro Ile Tyr Thr Asn Val Thr

120

Tyr Pro Ile Thr Val Asn Pro Pro Phe Val Pro Thr Glu Pro His

135

His His His Pro Gly Gly Arg Gln Met His Gly Tyr Asp Ala Glu

150

Leu Arg Leu Tyr Arg ↓Arg His His Gly Ser Gly Ser Pro Ser Arg

165

His Pro Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser

180

Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val

Lys Gly Arg Gly

Fig. 4

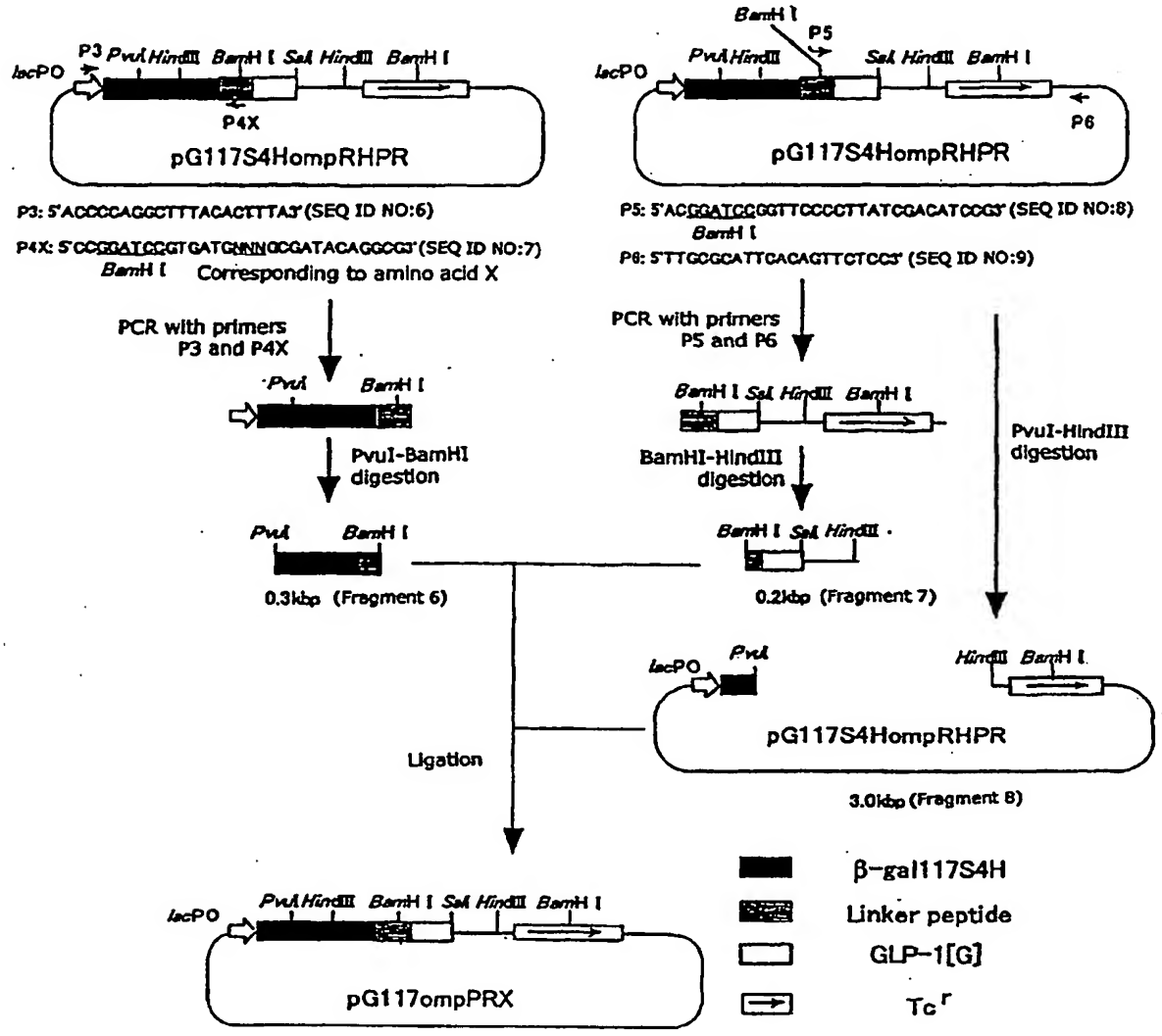


Fig. 5

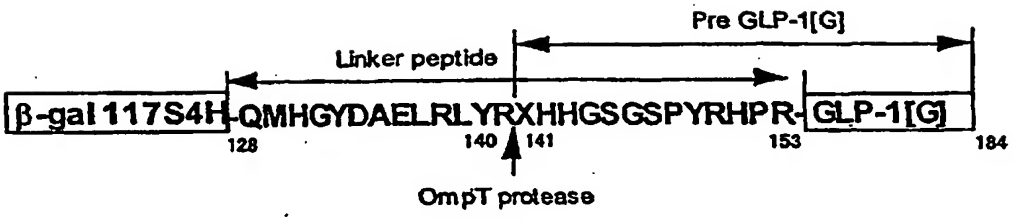


Fig. 6

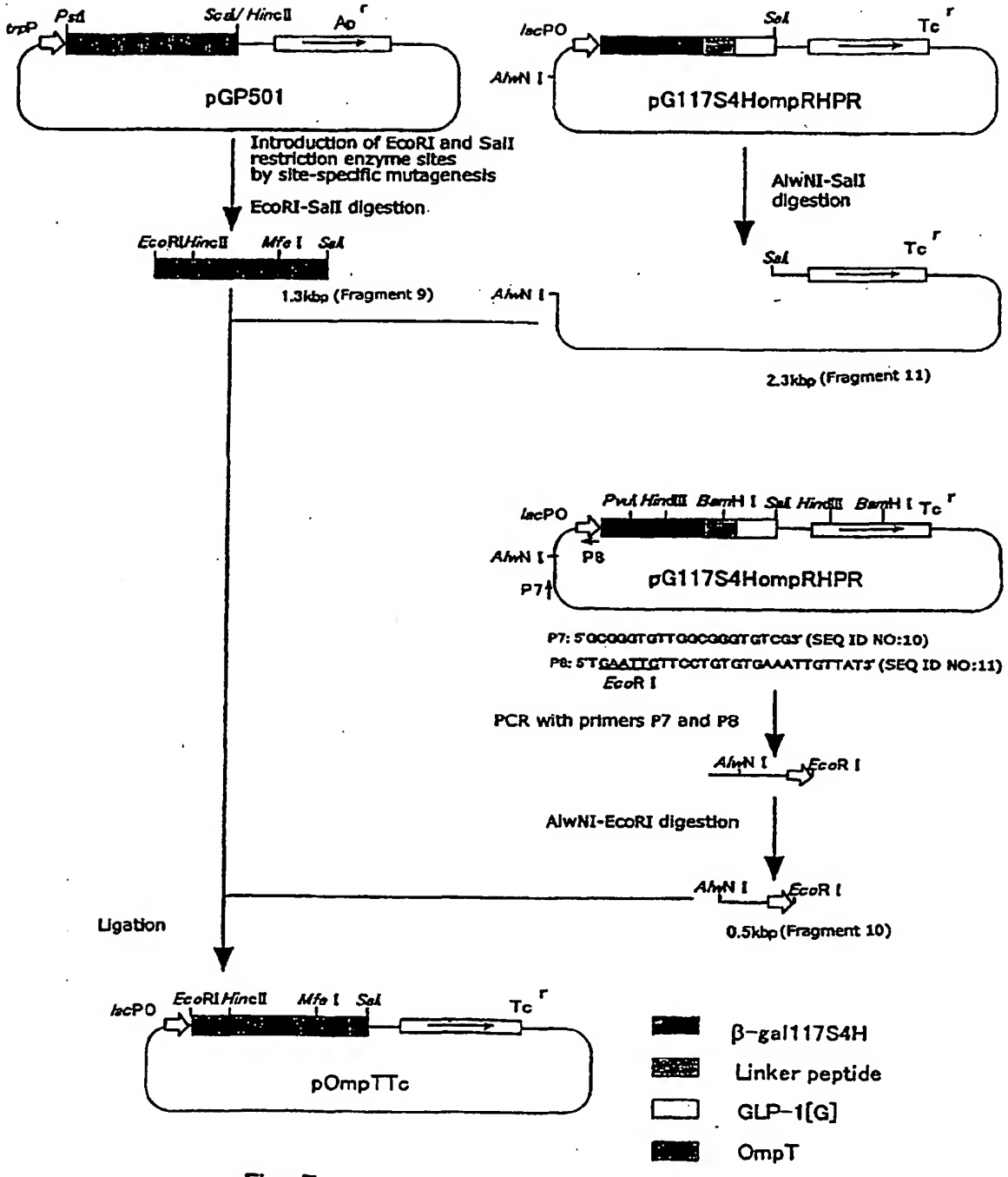


Fig. 7

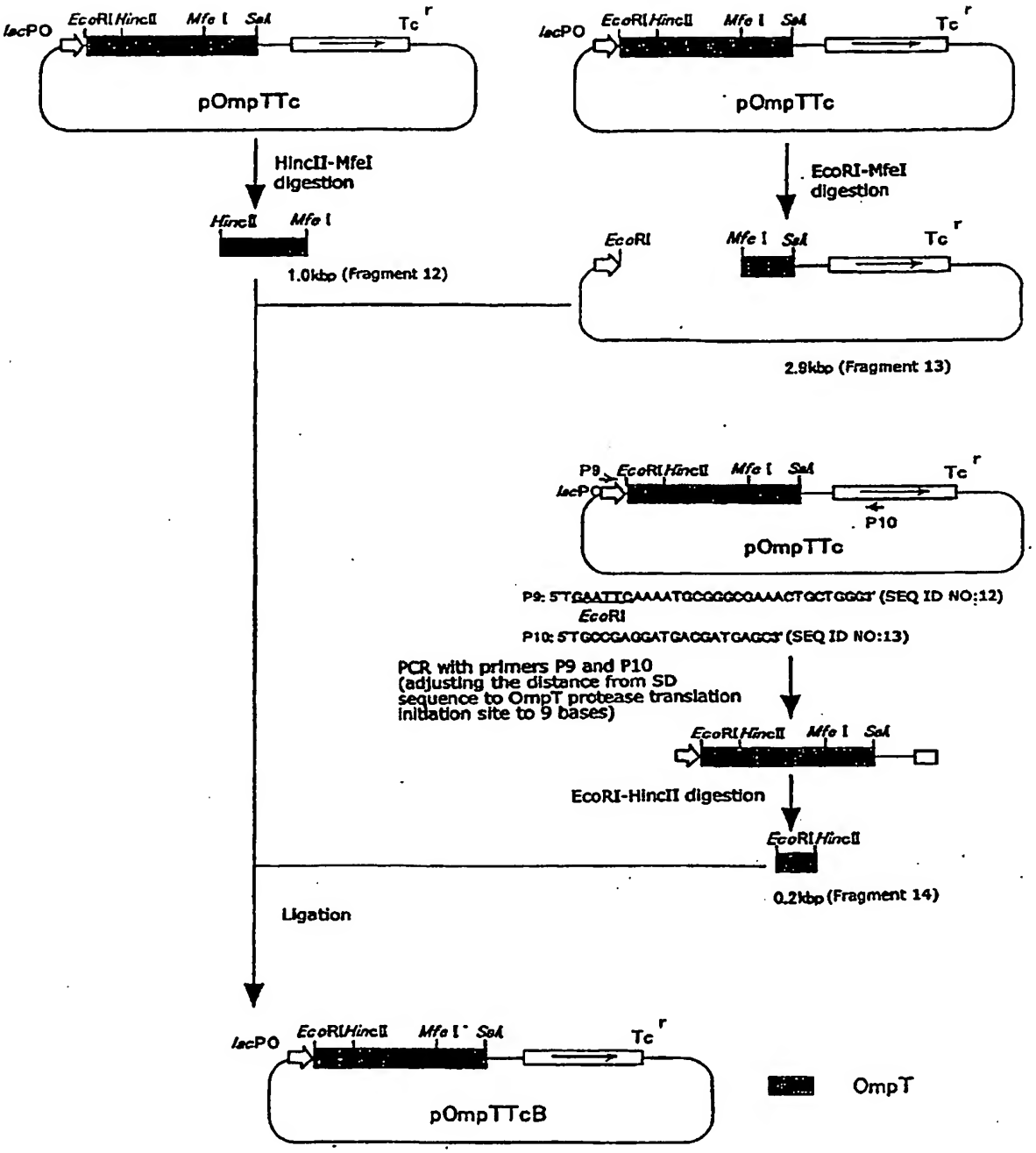


Fig. 8

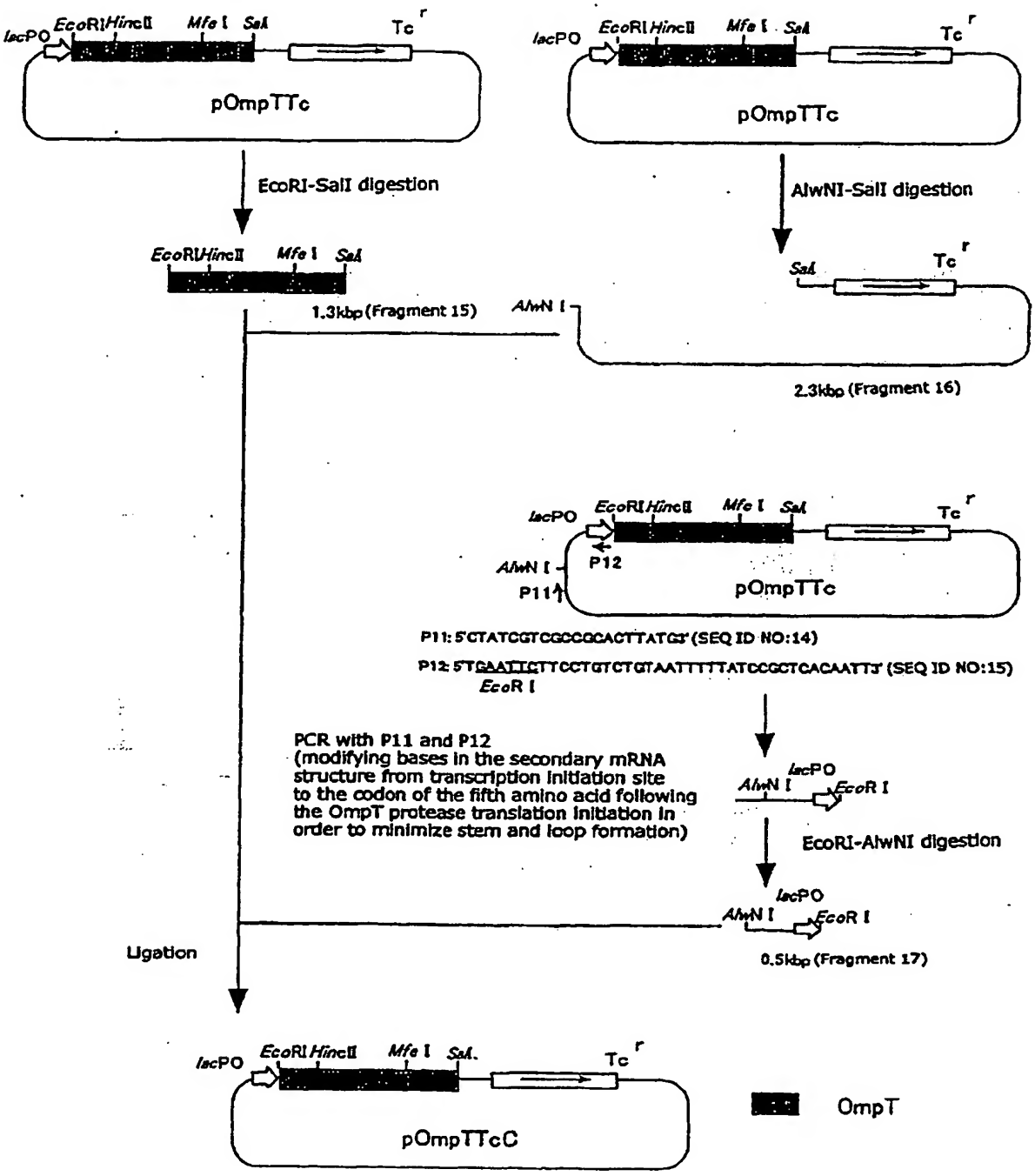


Fig. 9

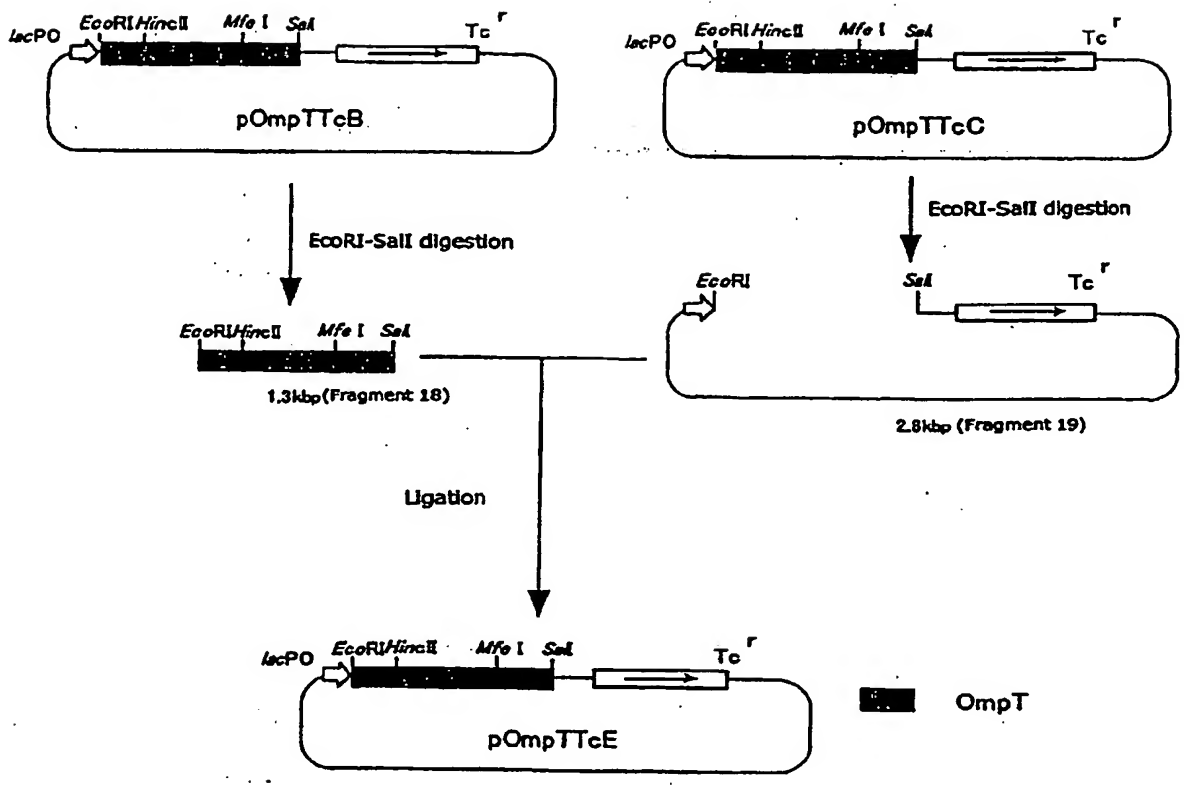


Fig. 10

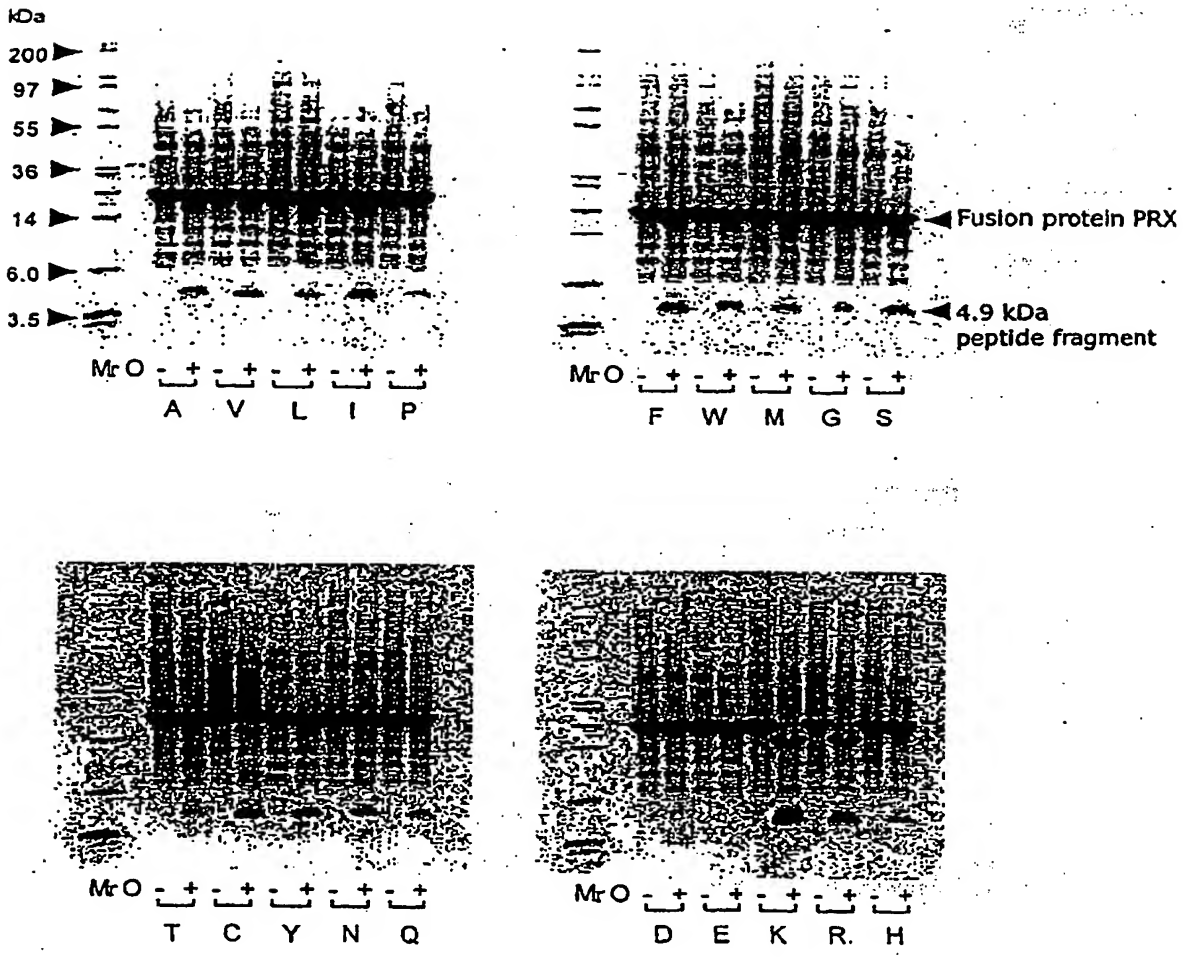

kDa
200
97
55
36
14
6.0
3.5
MrO
A V L I P
F W M G S
Fusion protein PRX
4.9 kDa
peptide fragment
T C Y N Q
D E K R. H

Fig. 11

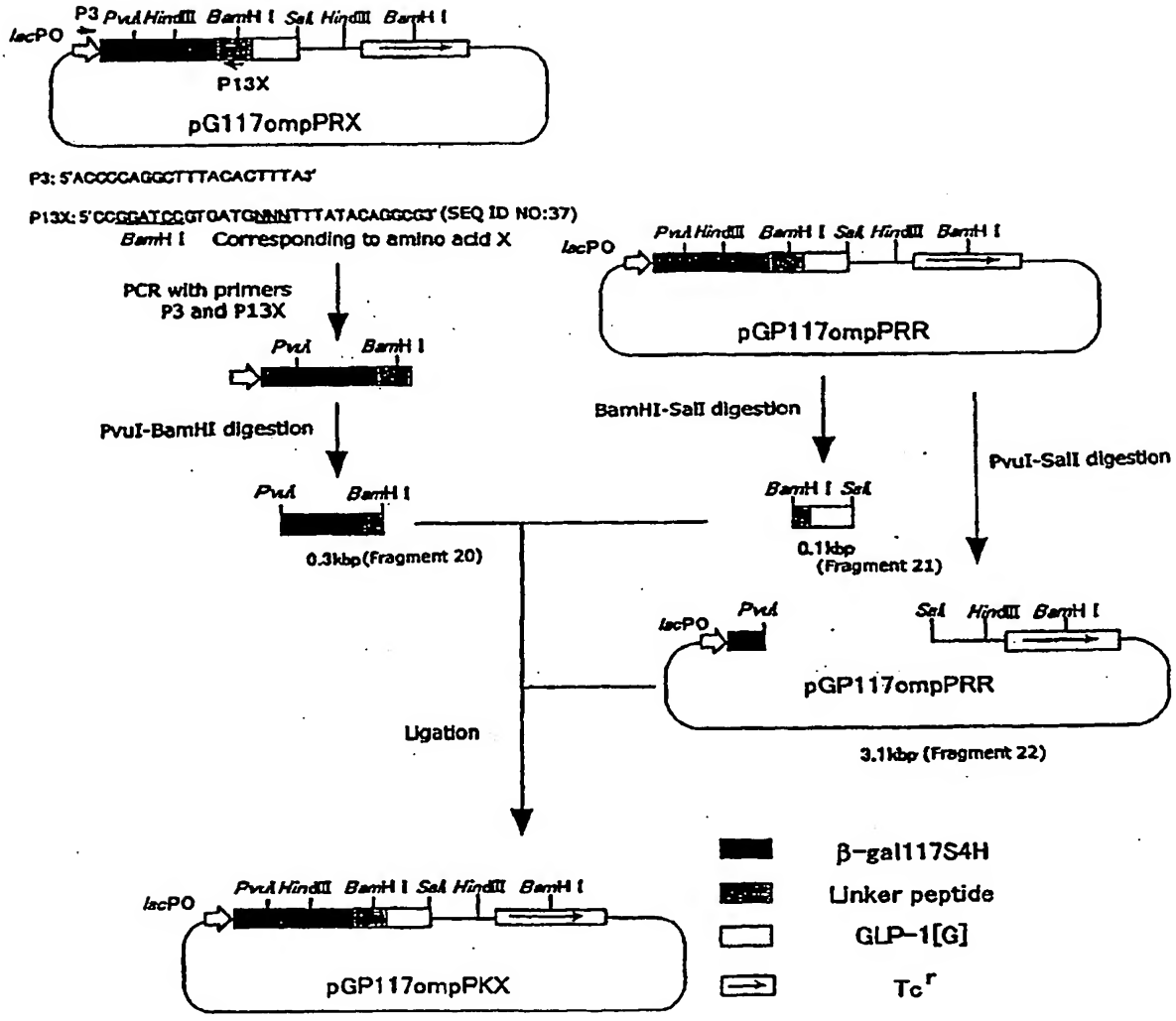


Fig. 12

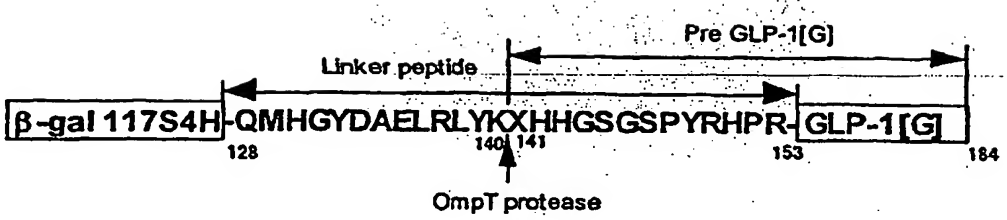


Fig. 13

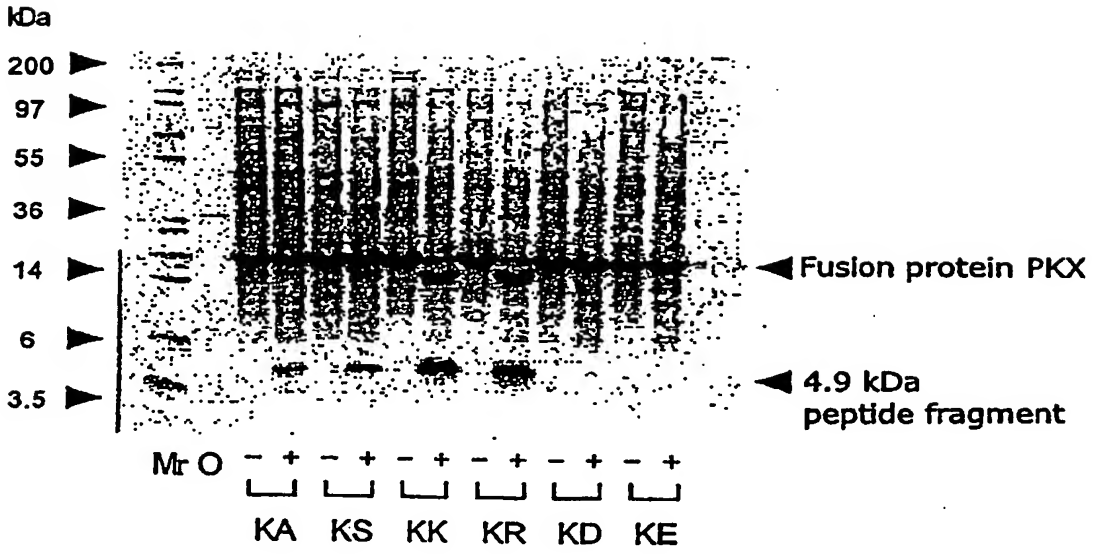


Fig. 14

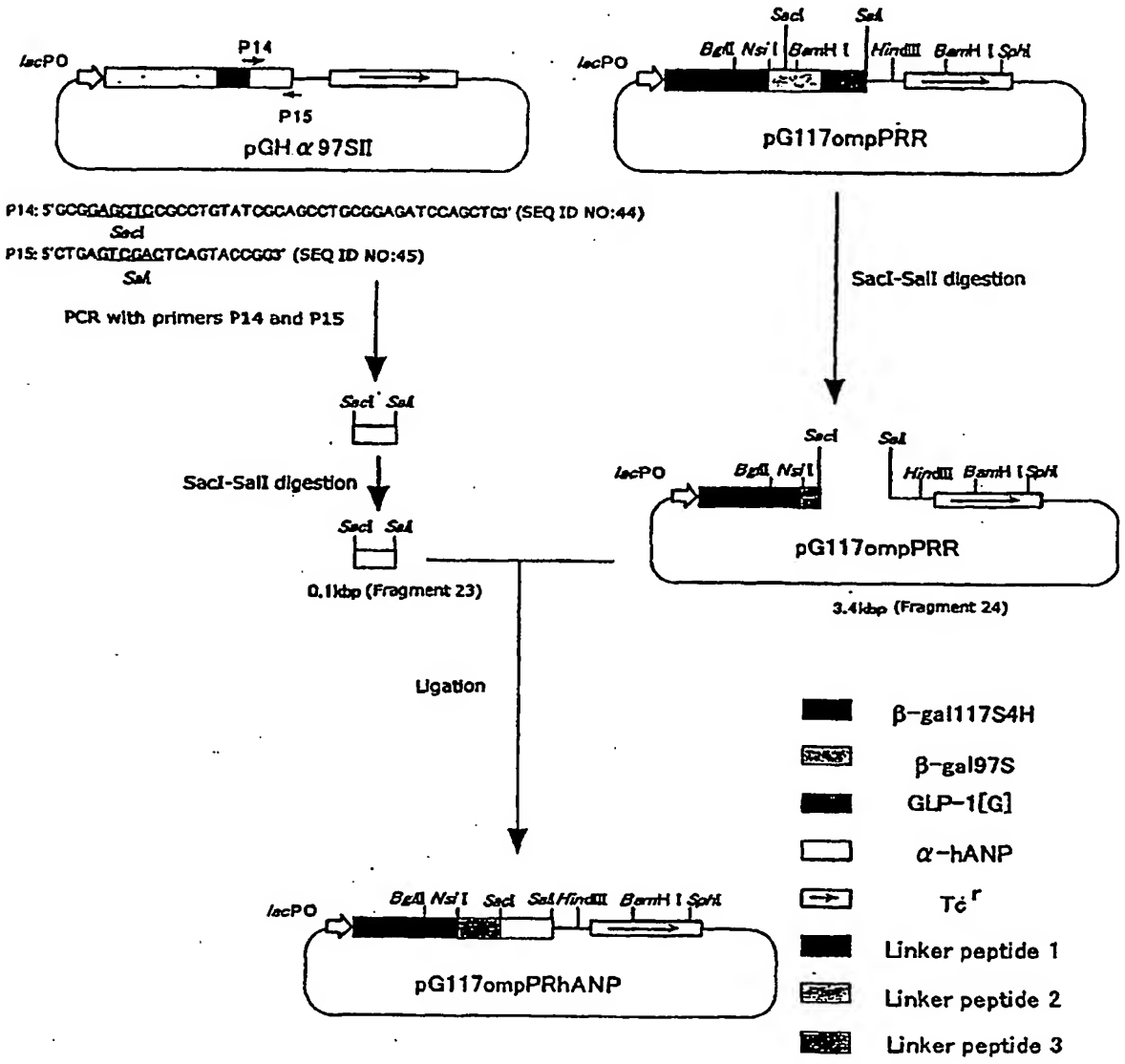


Fig. 15

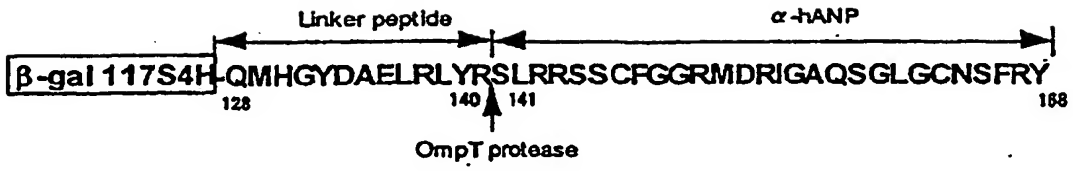


Fig. 16

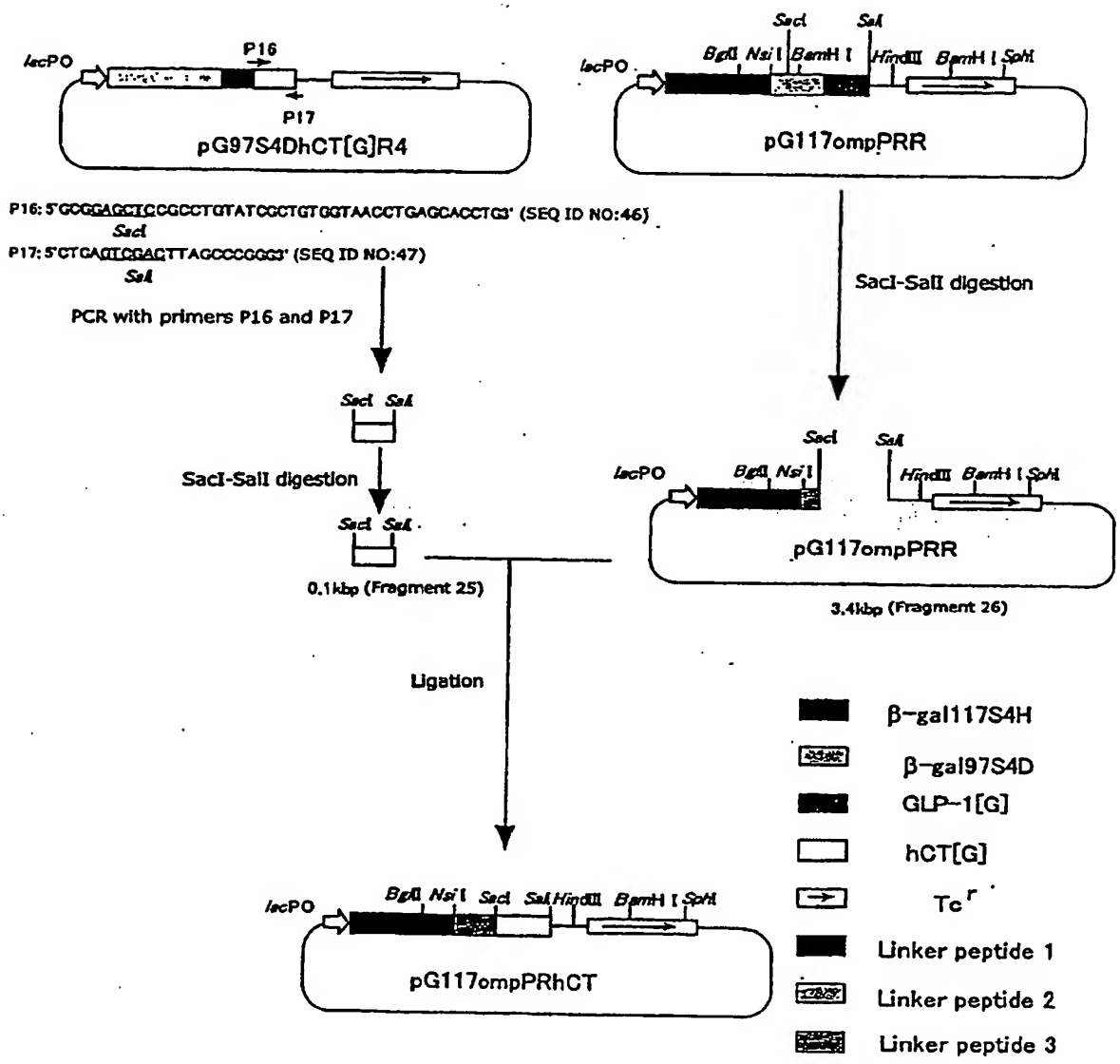


Fig. 17

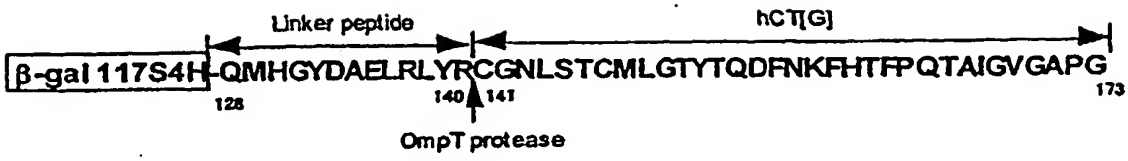


Fig. 18

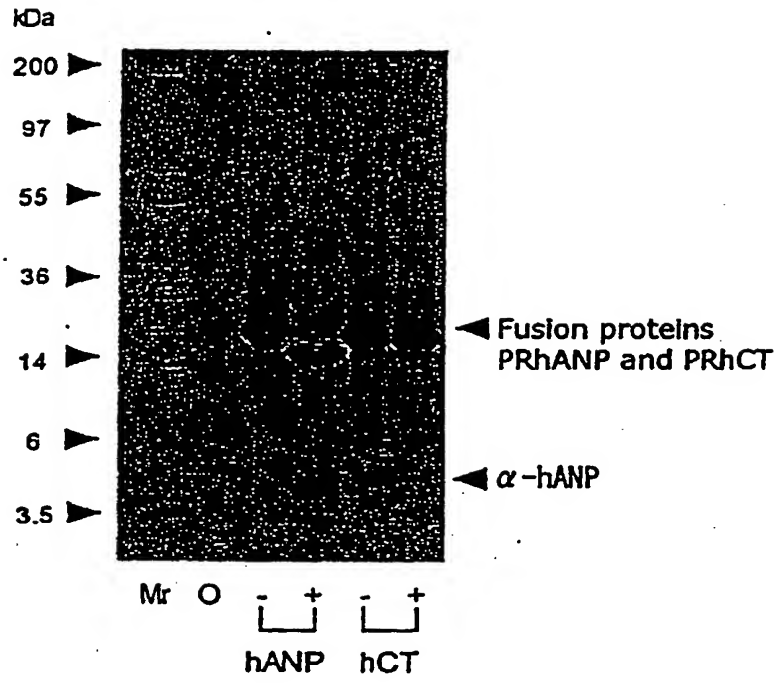


Fig. 19

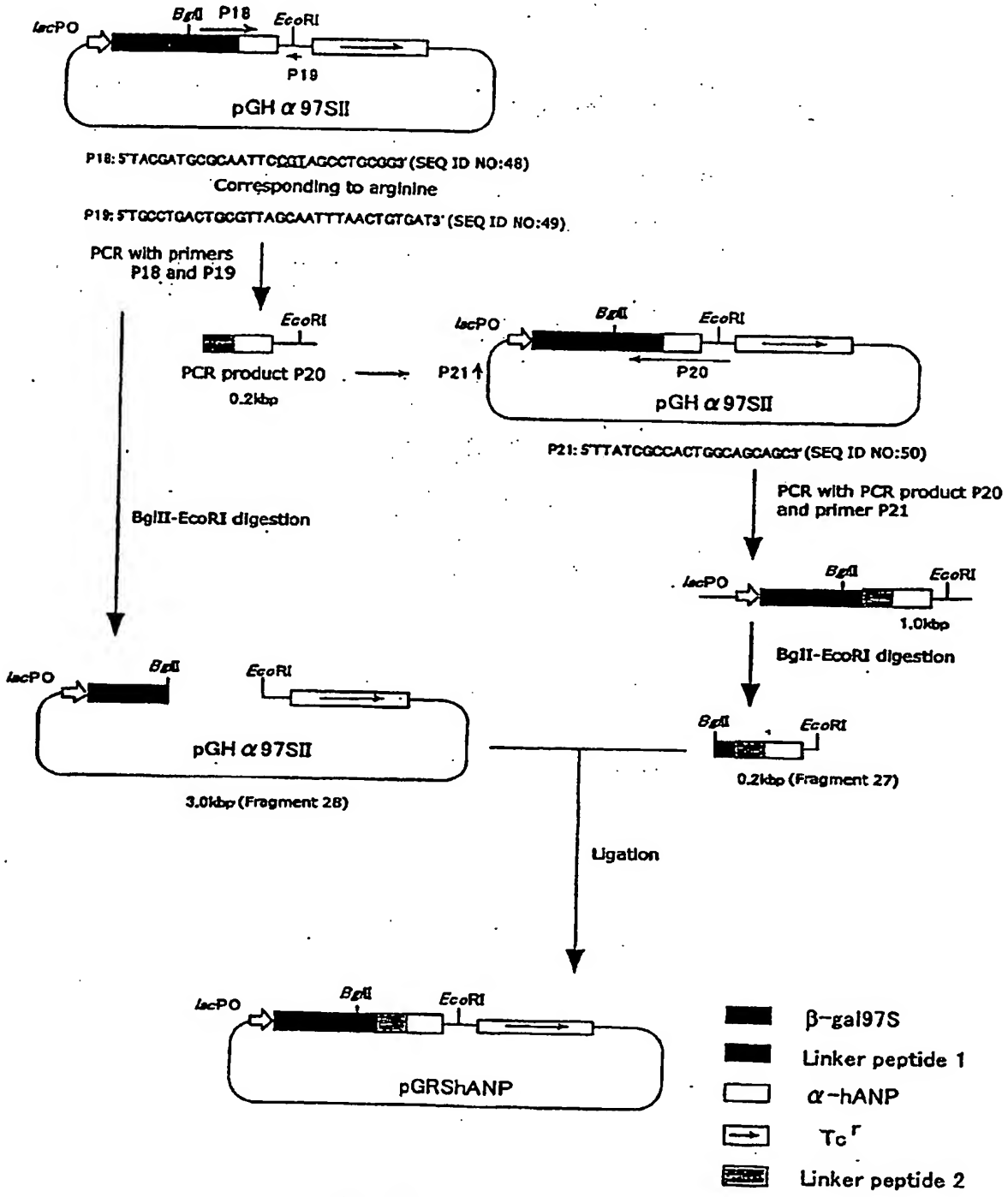


Fig. 20

15

Met Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Arg

30

Asp Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala

45

His Pro Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr

60

Asp Arg Pro Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg

75

Phe Ala Trp Phe Pro Ala Pro Glu Ala Val Pro Glu Ser Leu Leu

90

Glu Leu Pro Glu Ala Asp Thr Val Val Val Pro Asp Ser Ser Asn

105

Trp Gln Met His Gly Tyr Asp Ala Gln Phe Arg ↓ Ser Leu Arg Arg

120

Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser

Gly Leu Gly Cys Asn Ser Phe Arg Tyr

Fig. 21

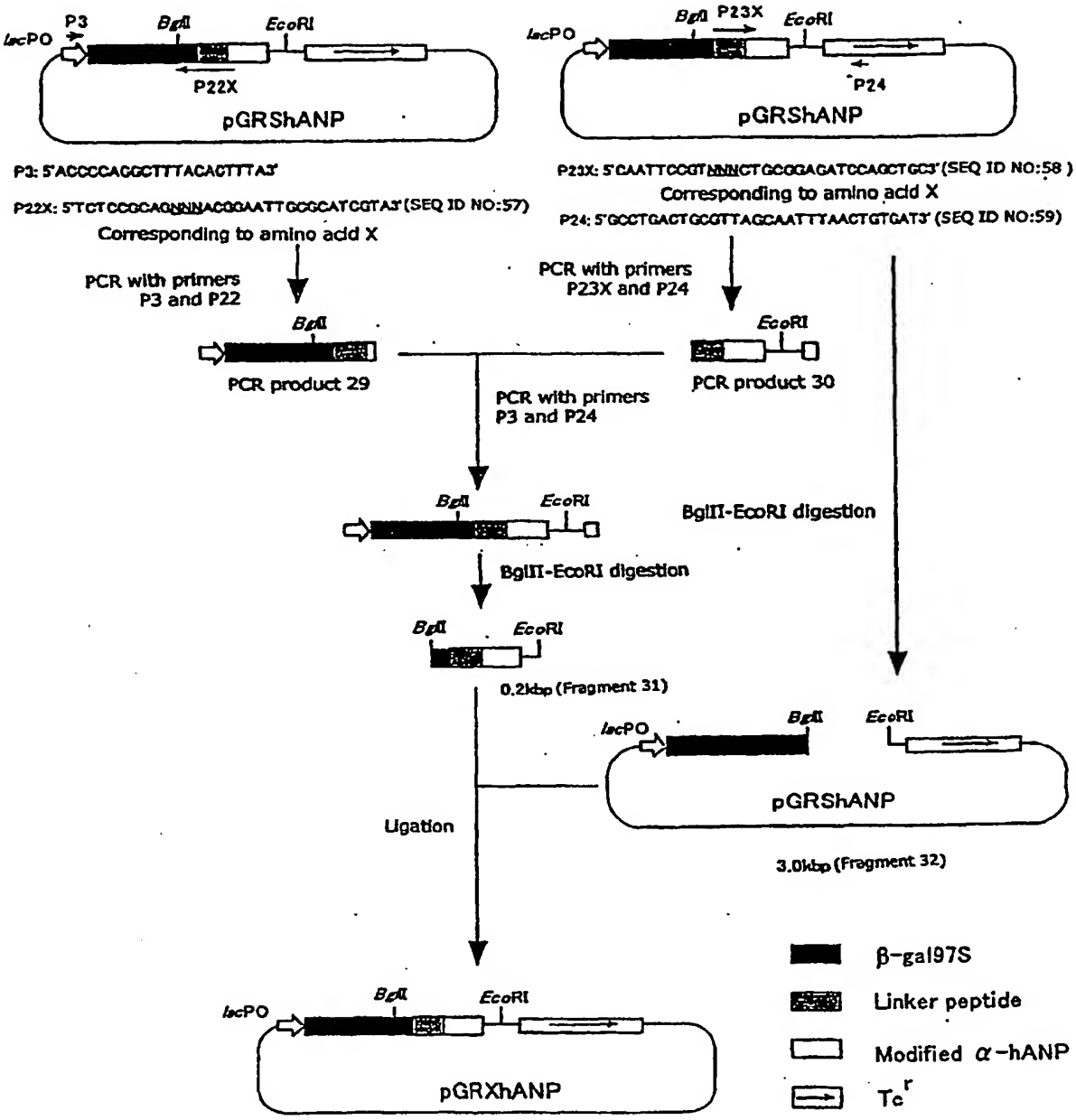


Fig. 22

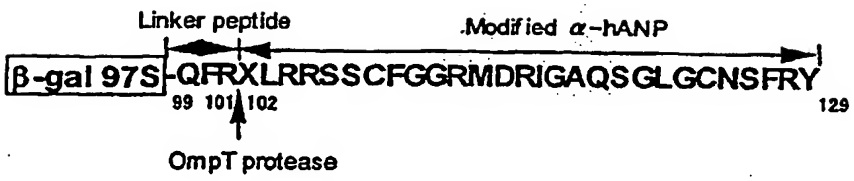


Fig. 23

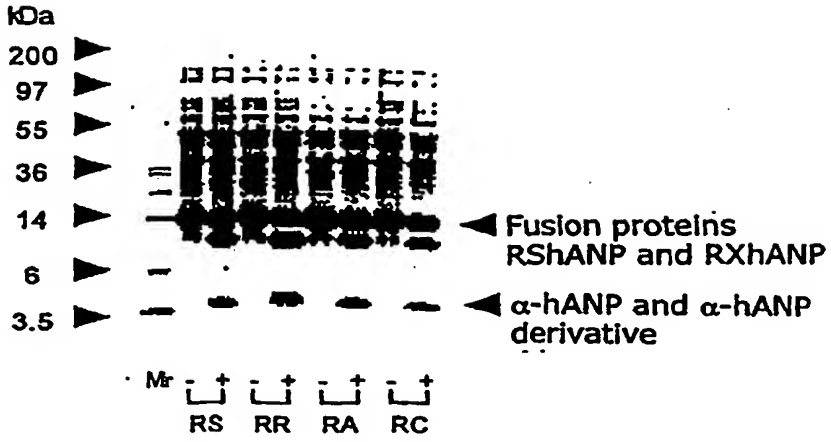
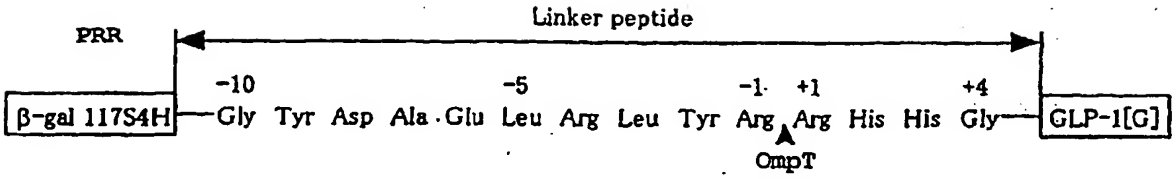


Fig. 24



PRR-1A	Gly	Tyr	Asp	Ala	Glu	Leu	Arg	Leu	Tyr	Ala	Arg	His	His	Gly
PRR-2A	Gly	Tyr	Asp	Ala	Glu	Leu	Arg	Leu	Ala	Arg	Arg	His	His	Gly
PRR-3A	Gly	Tyr	Asp	Ala	Glu	Leu	Arg	Ala	Tyr	Arg	Arg	His	His	Gly
PRR-4A	Gly	Tyr	Asp	Ala	Glu	Leu	Ala	Leu	Tyr	Arg	Arg	His	His	Gly
PRR-5A	Gly	Tyr	Asp	Ala	Glu	Ala	Arg	Leu	Tyr	Arg	Arg	His	His	Gly
PRR-6A	Gly	Tyr	Asp	Ala	Ala	Leu	Arg	Leu	Tyr	Arg	Arg	His	His	Gly
PRR-8A	Gly	Tyr	Ala	Ala	Glu	Leu	Arg	Leu	Tyr	Arg	Arg	His	His	Gly
PRR-9A	Gly	Ala	Asp	Ala	Glu	Leu	Arg	Leu	Tyr	Arg	Arg	His	His	Gly
PRR-10A	Ala	Tyr	Asp	Ala	Glu	Leu	Arg	Leu	Tyr	Arg	Arg	His	His	Gly

Fig. 25

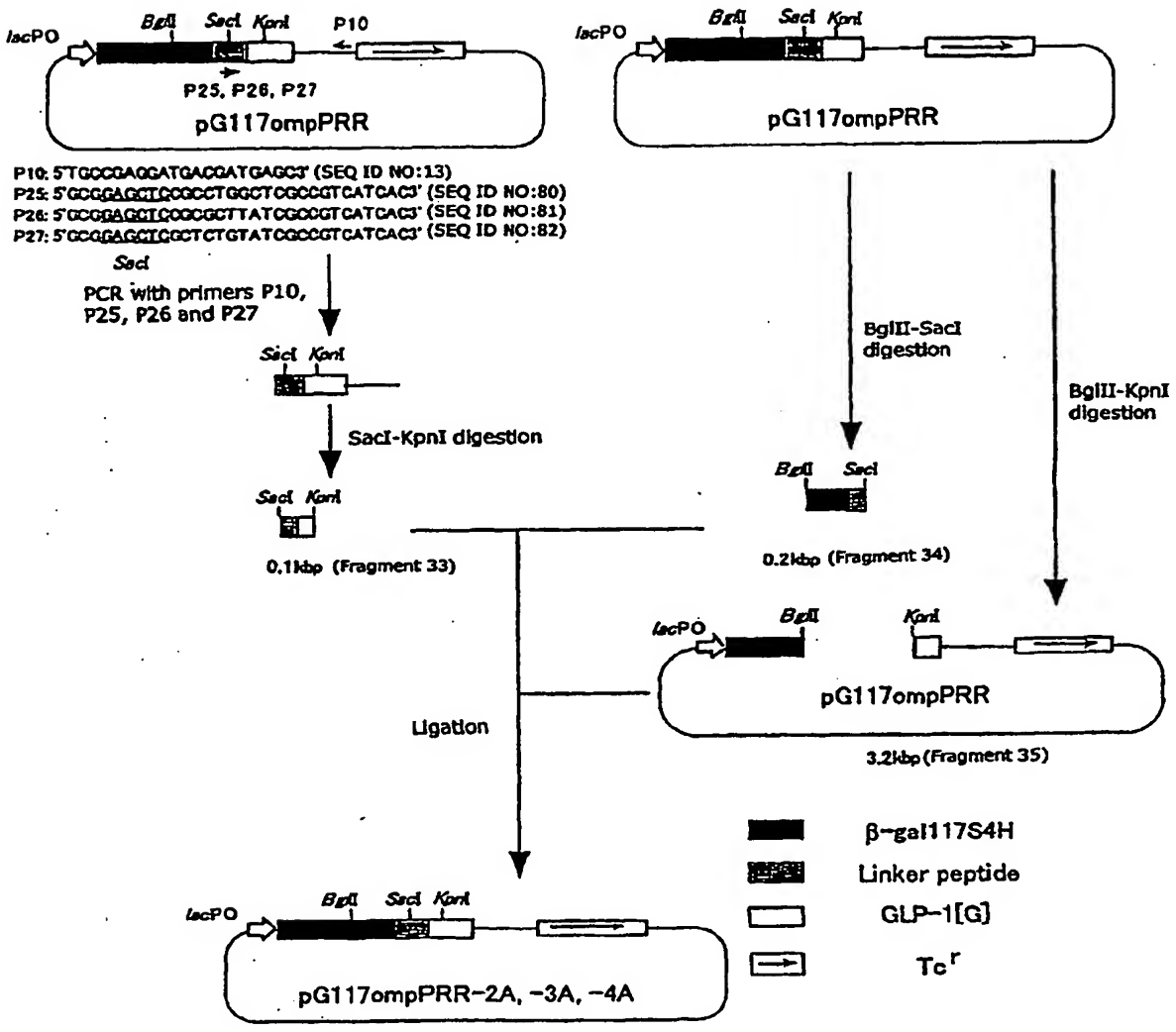


Fig. 26

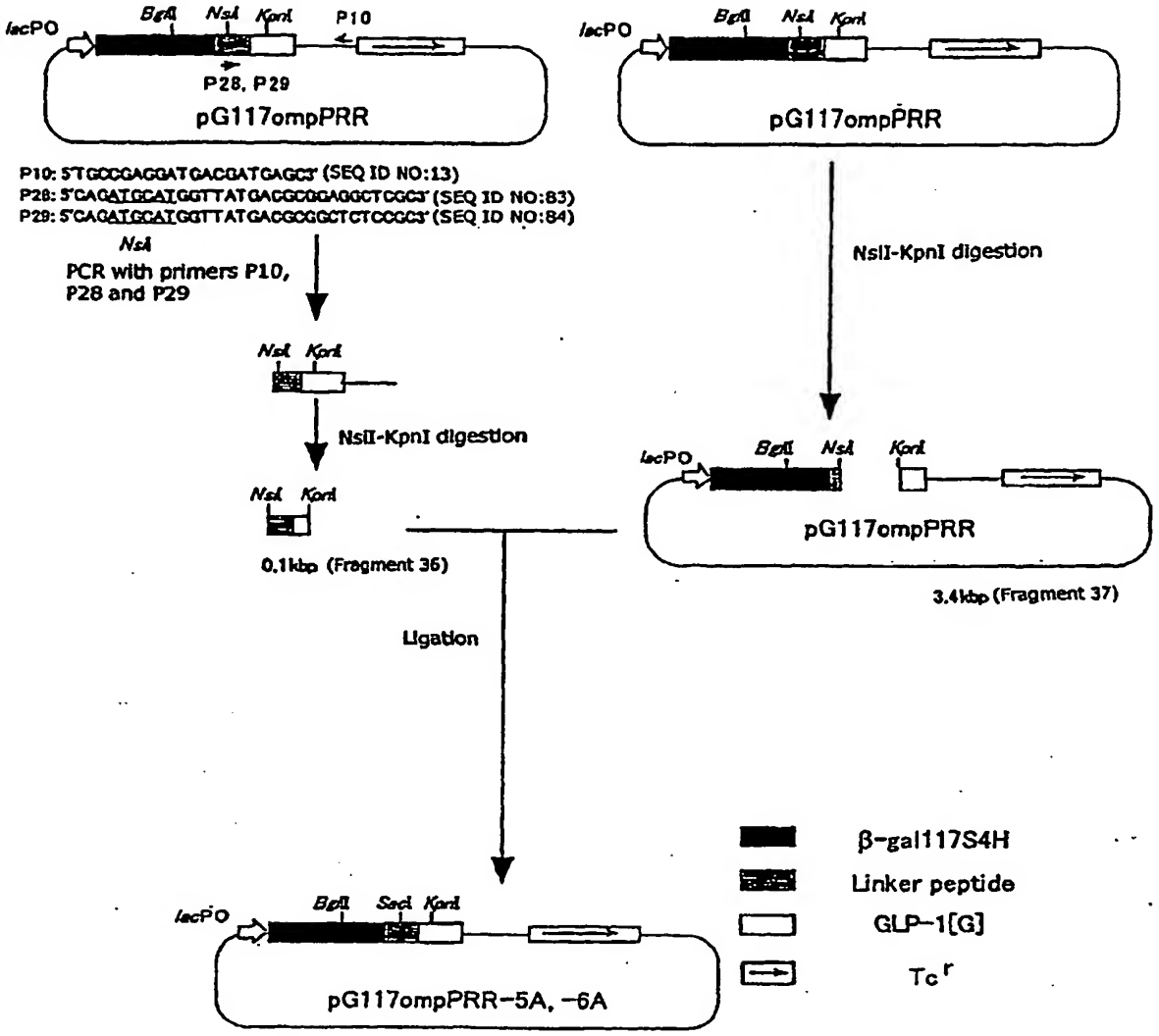


Fig. 27

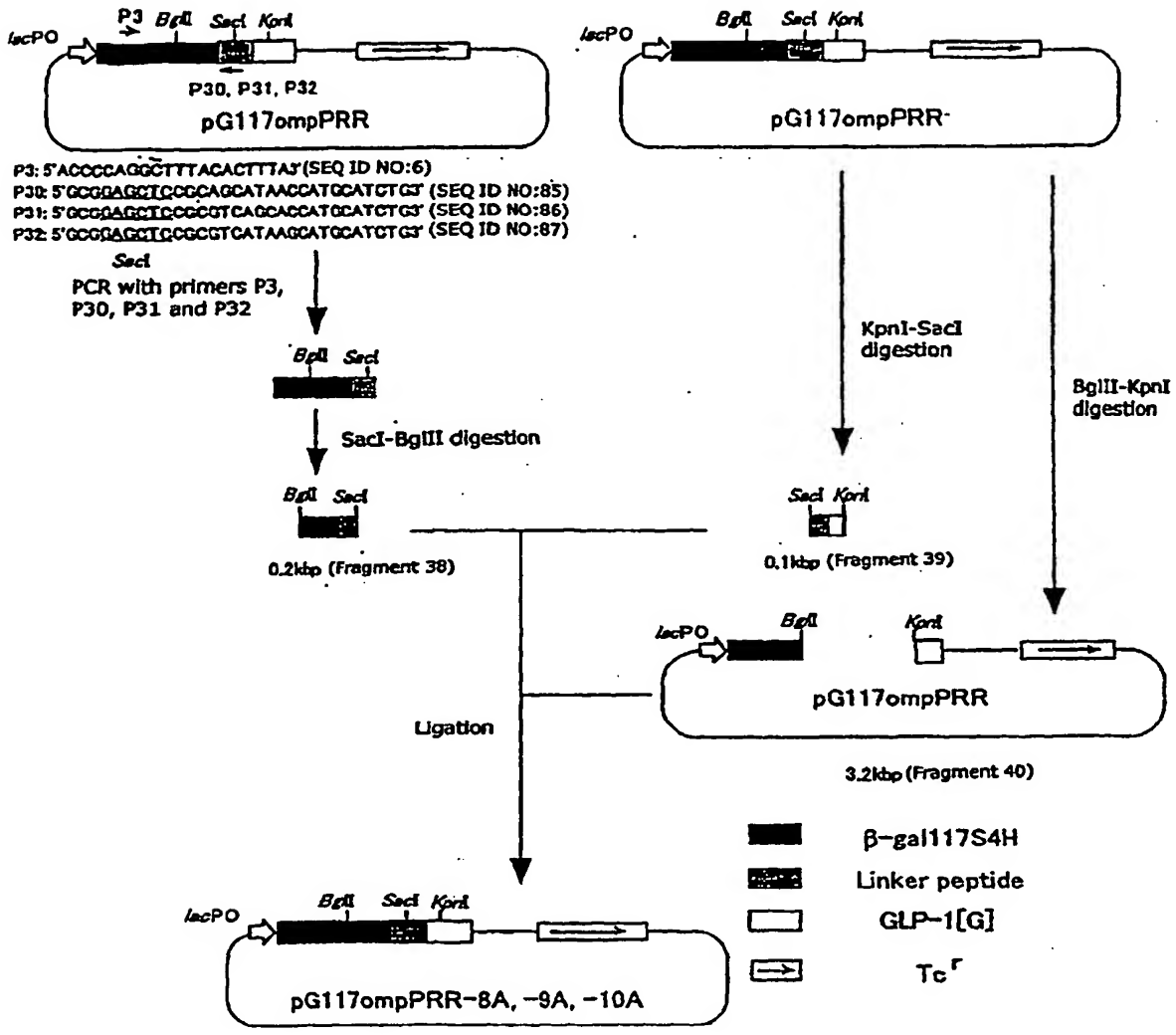


Fig. 28

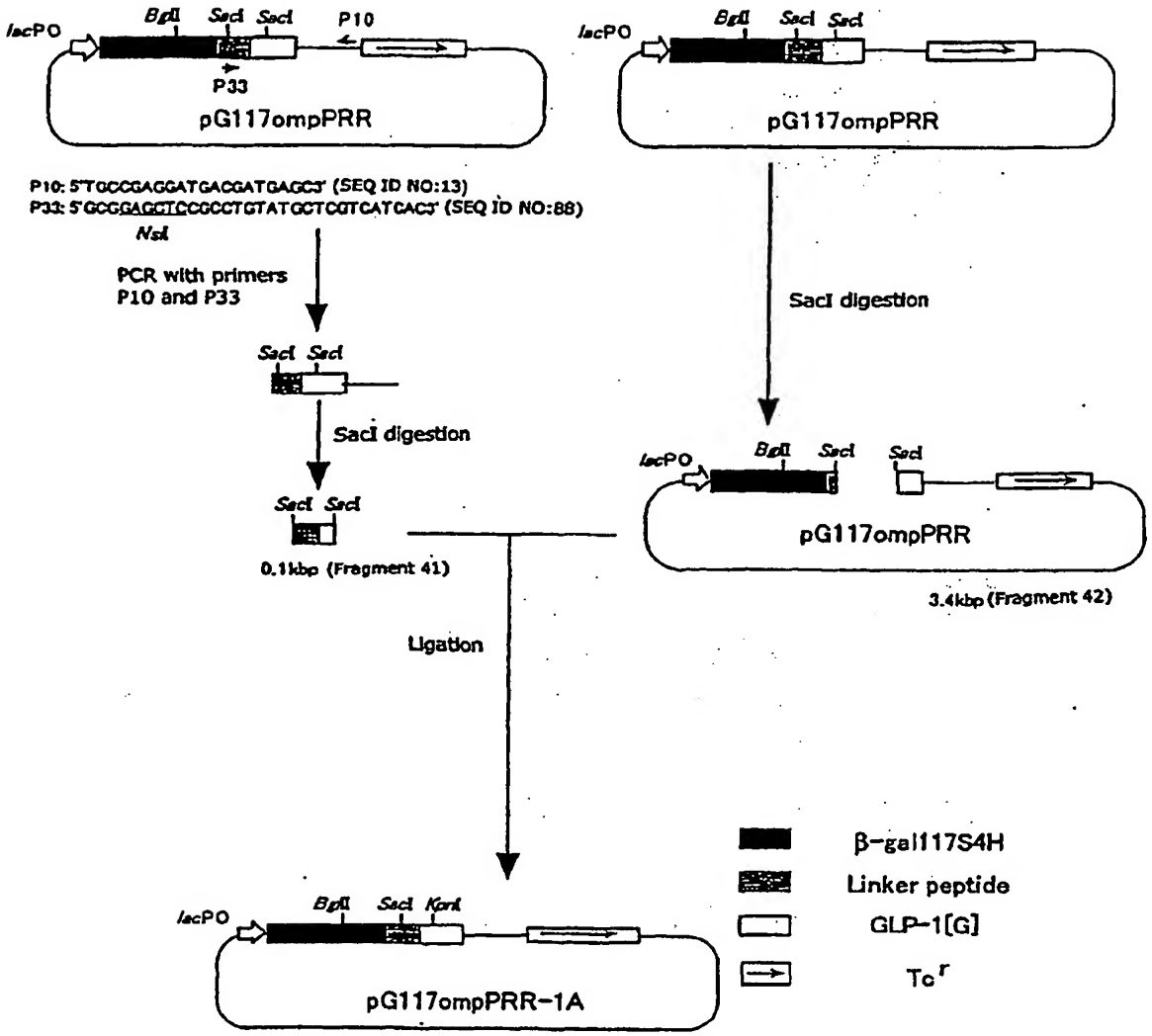


Fig. 29

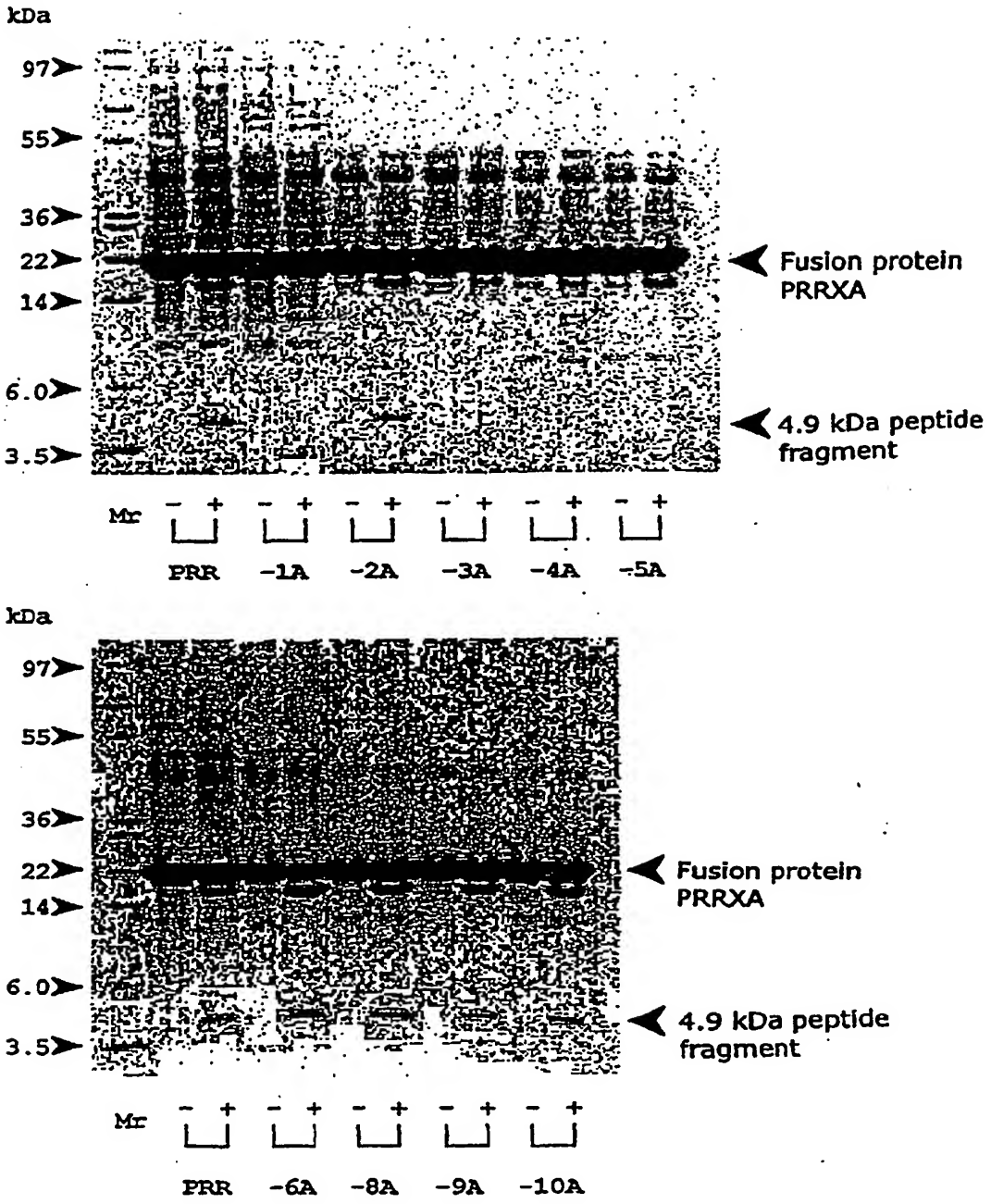


Fig. 30

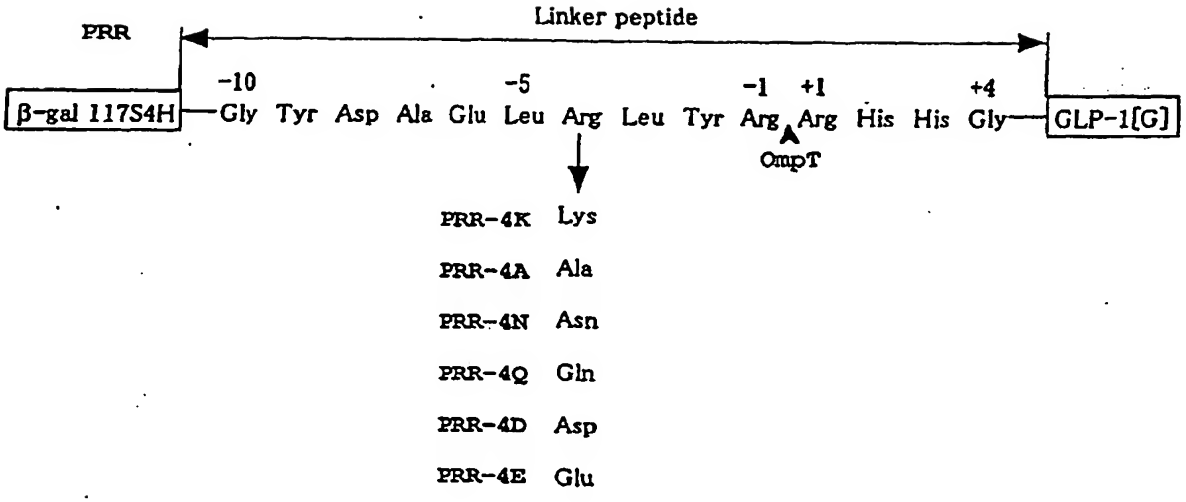

PRR
Linker peptide
β-gal 117S4H
-10
-5
-1
+1
+4
Gly Tyr Asp Ala Glu Leu Arg Leu Tyr Arg Arg His His Gly
GLP-1[G]
OmpT
PRR-4K Lys
PRR-4A Ala
PRR-4N Asn
PRR-4Q Gln
PRR-4D Asp
PRR-4E Glu

Fig. 31

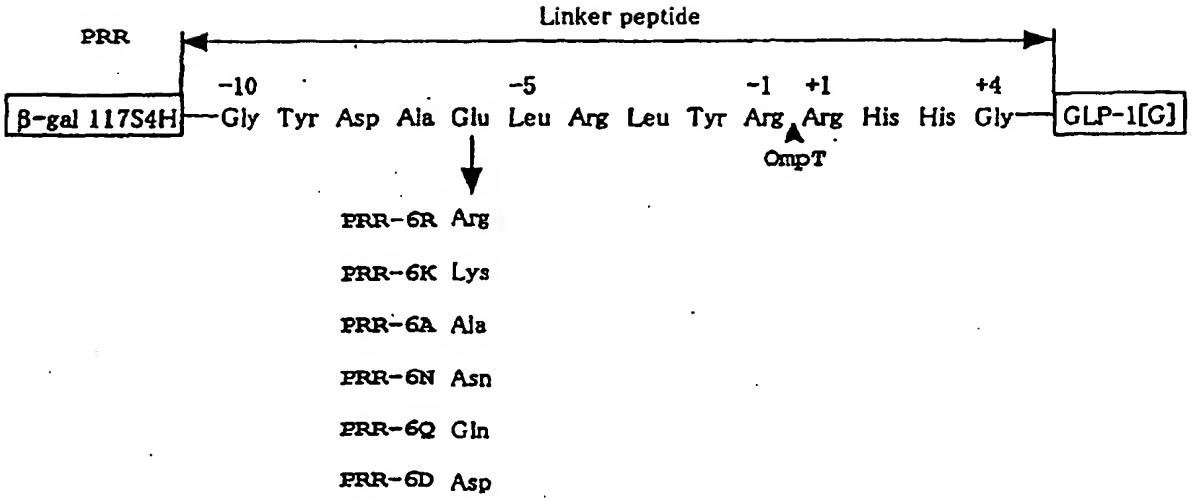


Fig. 32

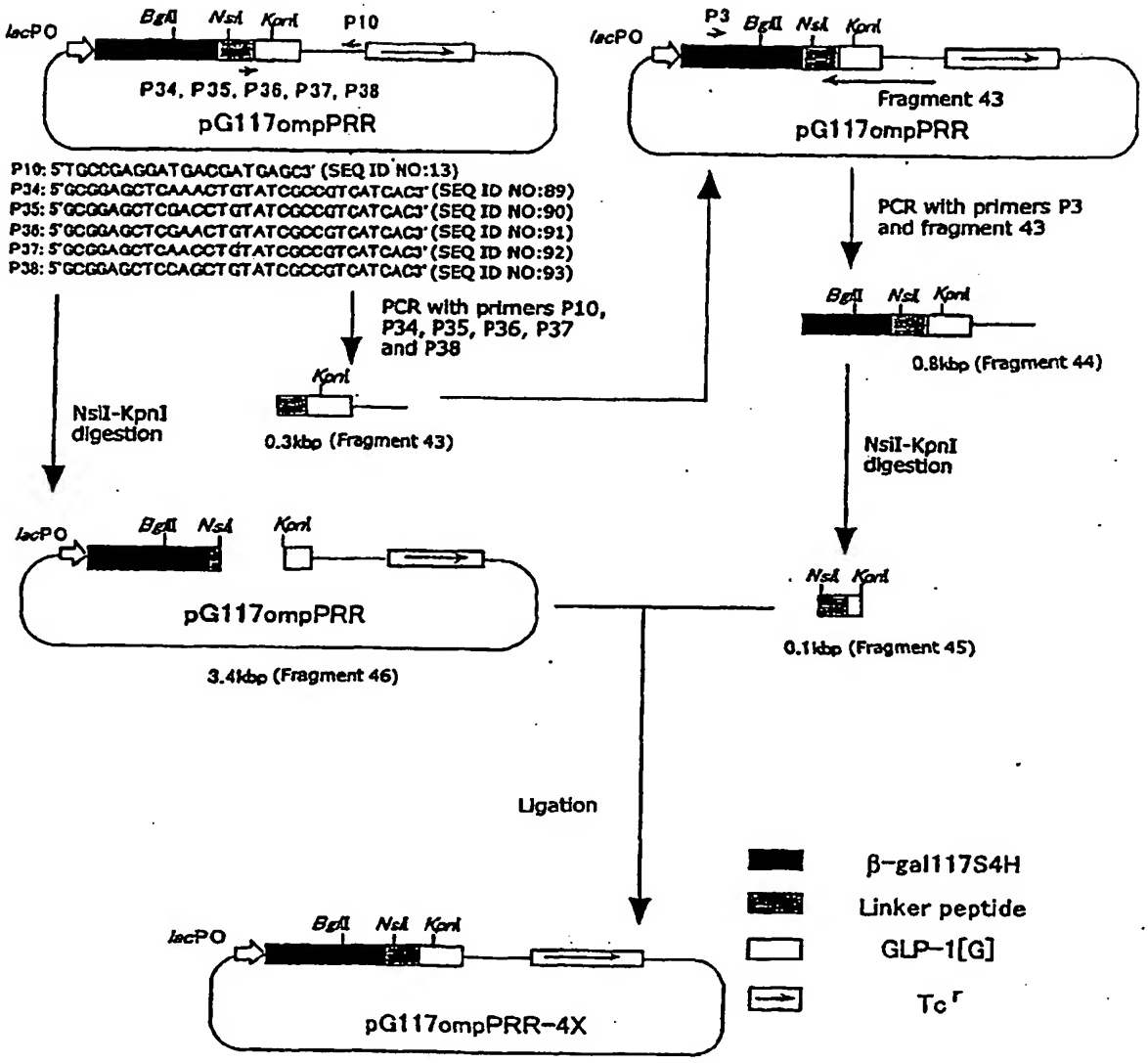


Fig. 33

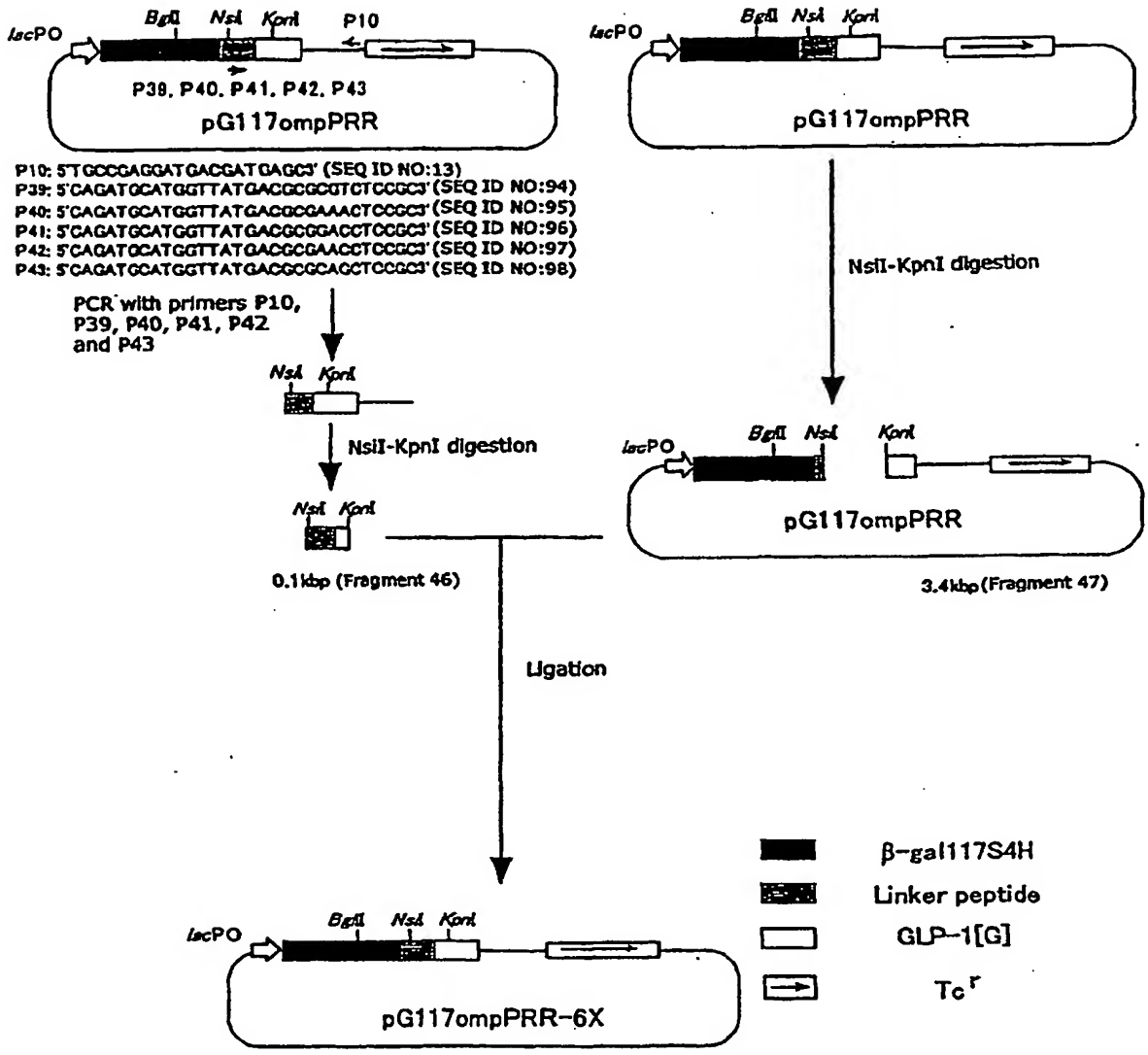


Fig. 34

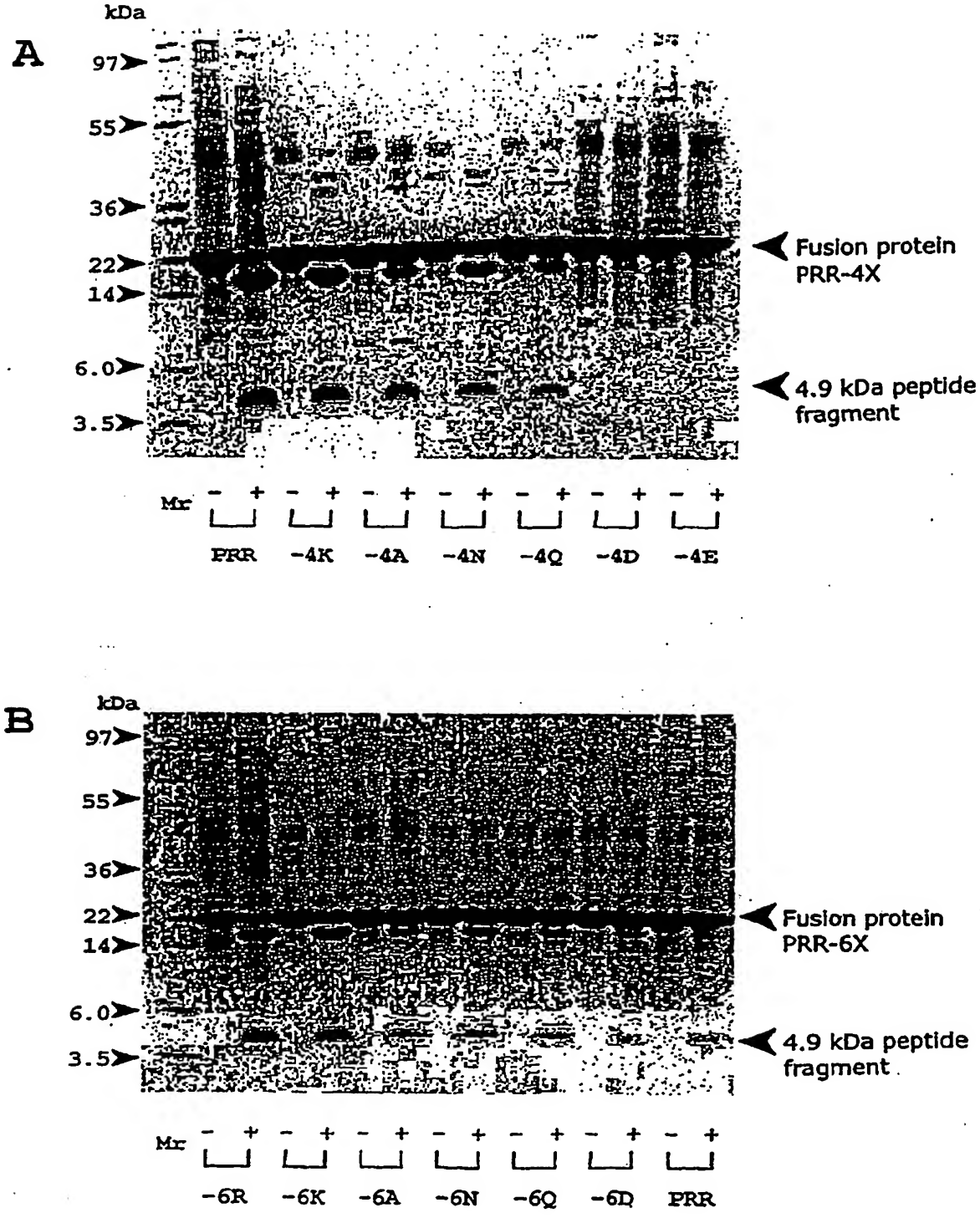


Fig. 35

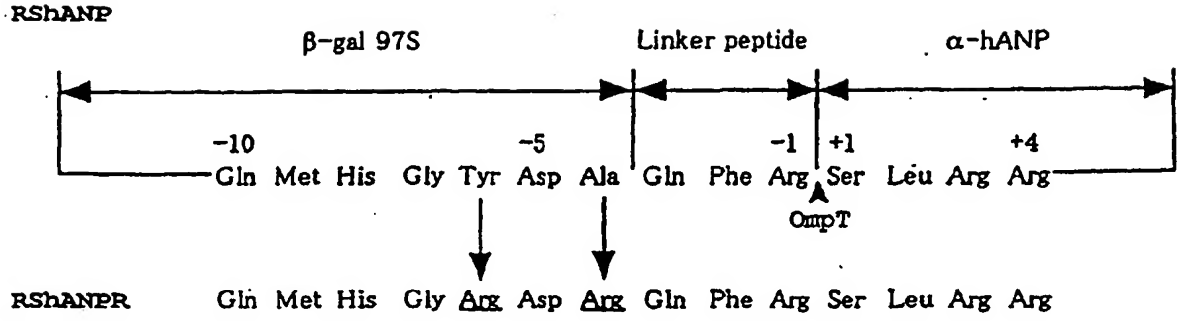


Fig. 36

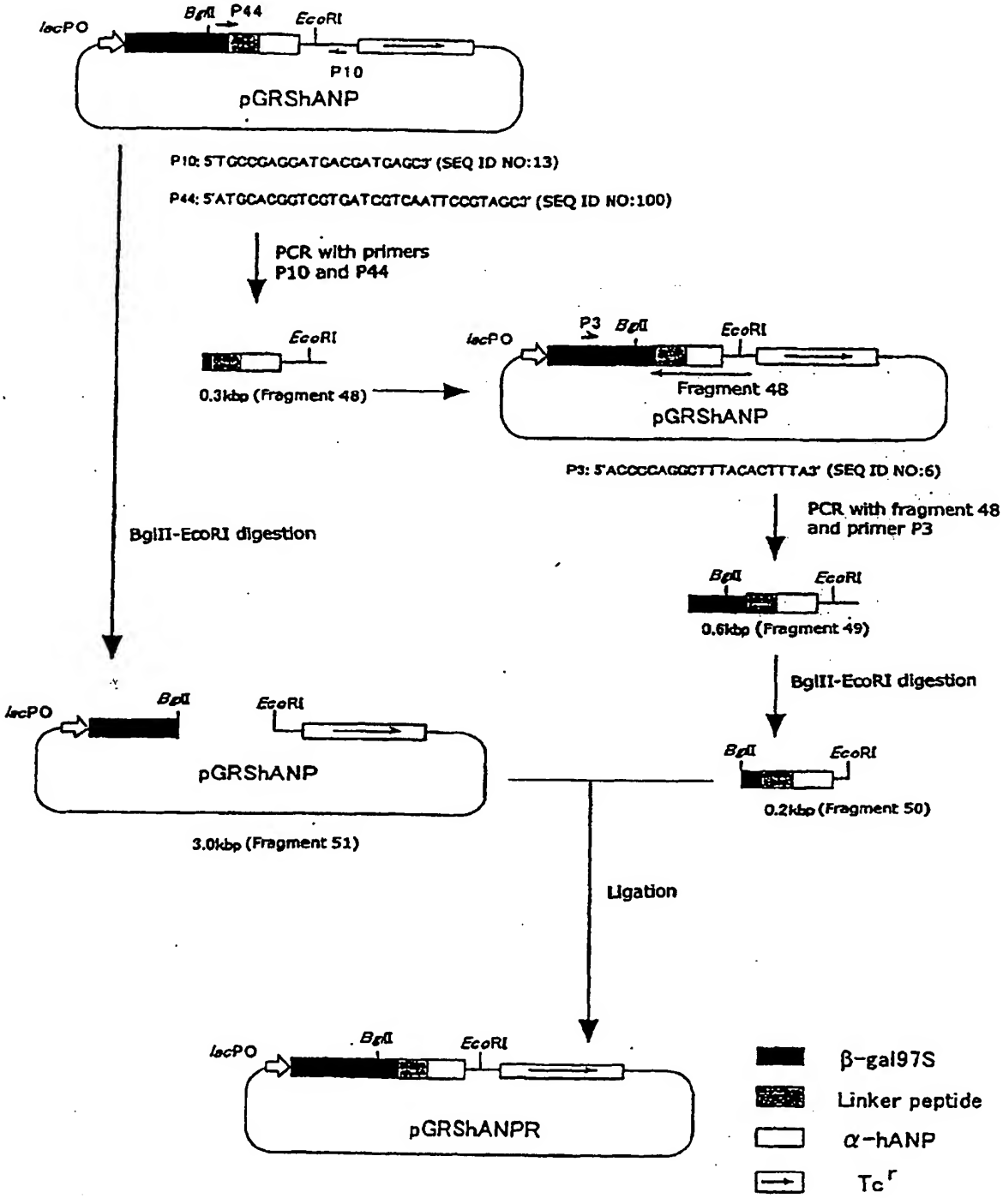


Fig. 37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/01309

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12P 21/02, C12N 15/09 // (C12P 21/02, C12R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12P 21/02, C12N 15/09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI, WPI/L, BIOSIS PREVIEWS, CAS ONLINE, GenBank/EMBL/DDBL/Geneseq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Yabuta M.et al. "Hyperproduction of a recombinant fusion protein of Staphylococcus aureus V8 protease in Escherichia coli and its processing by OmpT protease to release an active V8 protease derivative." Appl.Microbiol.Biotech., Vol.44, p.118-125 (1995)	1-16
A	Zhao Guo-Ping et al. "An amino acid switch (Gly-281 fwdarw Arg) within the hinge region of the tryptophan systhase beta subunit creates a novel cleavage site for the OmpT protease and selectively diminishes affinity toward a specific monoclonal antibody." J.Biol.Chem., Vol.268, No.20 p.14912-14920 (1993)	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May, 2000 (30.05.00)	20.06.00
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)